ENERGY-LINKED FUNCTIONS OF LYMPHOID TISSUE MITOCHONDRIA

by

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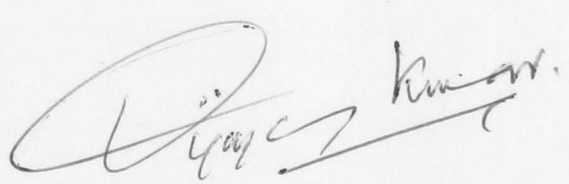
Submitted May, 1978

Submitted in satisfaction of the requirements for a degree of Doctor of Philosophy at the Australian National University, Department of Biochemistry, Faculty of Science, Canberra, Australia.

All of the results presented in this thesis on mitochondrial and sub-mitochondrial studies were my own work. A part of Chapter 2 (Fig. 2.1 - 2.3; Tables 2.1 - 2.4), on the quantitative aspects of glucose metabolism was done in collaboration with other workers in this laboratory. The adenine nucleotide exchange experiments were done in collaboration with the B.Sc (Hons) project of Mr I.A. Munro. Electron microscopic studies were carried out by Mr M.A. de Smet and Mr A. Pyliotis. The computer programme was prepared by Dr R.G. Ryall.



E.K. VIJAYAKUMAR

DEDICATED TO MY MOTHER

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TABLE OF CONTENTS

CHAPTER 3 LOCATION OF AN OLIGOMYCIN-INSENSITIVE AND MAGNESIUM PLUS CALCIUM ION-STIMULATED ADENOSINE TRIPHOSPHATASE IN RAT SPLEEN MITOCHONDRIA

CHAPTER 4 KINETIC PROPERTIES OF THE ATPase FROM THE OUTER MEMBRANE OF RAT SPLEEN MITOCHONDRIA

CHAPTER 5 POSSIBLE FUNCTIONAL ROLE OF THE OUTER MEMBRANE ATPase OF RAT SPLEEN MITOCHONDRIA

LIST OF FIGURES AND PLATES

LIST OF TABLES

ACKNOWLEDGEMENTS

I thank Professor J.F. Williams for allowing me the privilege of studying for this degree in the warm and friendly atmosphere of his Biochemistry department. I am extremely grateful to my supervisor Dr M.J. Weidemann for his constant interest, both in my project and in my personal life, encouragement and advice during the entire period of this work. Many helpful discussions with Fyfe Bygrave, Alan Laird, David Hume, Ian Munro and Lesley Russell are gratefully acknowledged. My thanks also go to Agu Majajas for preparation of photographs, and to Kerry Navin for the typescript. A very special thanks go to my wife Shan for her tremendous help and patience in preparing the whole manuscript.

This investigation was supported financially by an Australian National University Scholarship and in part from an ARGC grant (D 2 70/17436) provided by Dr M.J. Weidemann.

ABSTRACT

1. Addition of the plant lectin concanavalin A to rat thymus lymphocytes stimulates (by 2 fold) the uptake of [U-^{14}C]-glucose over 3 hrs and causes a concomitant increase in the incorporation of glucose carbon into protein, lipids and RNA. A large percentage of the extra glucose taken up is converted, under aerobic conditions, into lactate; concanavalin A also causes a specific increase in pyruvate oxidation, leading to an increase in the percentage contribution made by glucose to the respiratory fuel.

2. Acetoacetate oxidation, which is not affected by concanavalin A, strongly suppresses the oxidation of pyruvate formed in the presence of glucose, but does not prevent the concanavalin A-induced stimulation of this process. Thus, whilst the addition of acetoacetate does not affect the uptake of glucose, it strongly increases the conversion of glucose into lactate. Alterations in glucose uptake and pyruvate oxidation appear to be the major metabolic consequences of lectin stimulation.

3. When isolated rat lymphoid tissue (spleen and thymus) mitochondria are incubated at Ca^{2+} concentrations in the nanomolar range, this causes a specific stimulation of the oxidation of pyruvate (and 2-oxoglutrate). This observation is consistent with the stimulation of pyruvate oxidation observed in lymphocytes treated with concanavalin A or the calcium ionophore A23187, which both enhance cytosolic Ca^{2+} levels.

4. Higher concentrations of free Ca^{2+} (>50nM) inhibit the oxidation of NAD-linked substrates by lymphoid tissue mitochondria without affecting the oxidation of succinate or ascorbate-TMPD.

5. Addition of free Mg^{2+} (150μM) stimulates markedly the state-4 respiration rate of spleen mitochondria and abolishes both the respiratory control and ADP/O ratios; this effect is reversible on addition of excess EDTA. Thymus mitochondrial oxidation, which is relatively unaffected at

this low concentration of free Mg^{2+}, does change to some extent at higher concentrations (>3mM).

6. With [γ^{32}P]-ATP as substrate, a Mg^{2+}-stimulated ATPase has been identified in the atractyloside-insensitive and EDTA-accessible space of intact spleen mitochondria. Oligomycin has no effect on the activity of this enzyme at a concentration (2µg/mg of protein) that completely inhibits the atractyloside-sensitive reaction (i.e., ATP-synthase activity).

7. On digitonin fractionation, the atractyloside- and oligomycin-insensitive, Mg^{2+}-stimulated ATPase co-purifies with the outer membrane sub-fraction of spleen mitochondria, whereas the atractyloside-sensitive activity co-purifies with the inner membrane plus matrix sub-fraction. Preliminary studies suggest that the outer membrane ATPase is present in mitochondria from rat kidney-cortex and heart as well as from spleen, but is absent from rat liver, brain, lung, diaphragm and skeletal muscle. Thymus mitochondria possess a lower activity than that of spleen.

8. The outer membrane ATPase, which is stable on storage in liquid N_2, is inactive unless Mg^{2+} is added (K_m for MgATP = 50µM). The substrate for this enzyme is a bivalent metal ion-nucleoside triphosphate complex in which Mg^{2+} (K_m = 50µM) can be replaced effectively by Ca^{2+} (K_m = 6.7µM) or Mn^{2+}, and ATP by ITP. Other bivalent cations (e.g., Co^{2+}, Ba^{2+}, Zn^{2+}, Ni^{2+}, etc.) support very little ATP hydrolysis.

9. The Mg^{2+}-dependent ATPase activity is enhanced (<10%) by low concentrations (50mM) of univalent cations (e.g., Na^+, K^+, Rb^+, Cs^+, NH_4^+, etc.) which are inhibitory (20-30%) at higher concentrations (500mM). The activity is inhibited significantly by Cu^{2+} (K_i = 90µM), Ni^{2+} (K_i = 510µM) and Co^{2+} (K_i = 1020µM), but not by Mg^{2+}, Ca^{2+}, Ba^{2+} or Sr^{2+}. The activity is insensitive to the inhibitors oligomycin, N,N'-dicyclohexylcarbodiimide, NaN_3, ouabain and thiol-specific reagents. A large variety of metabolic intermediates also have no significant inhibitory effects. A significant

inhibition is observed at higher concentrations of $AgNO_3$ (0.5mM) and NaF (10mM). The activity is inhibited competitively by the product MgADP (K_i = 0.7mM) but not by the second product P_i or by 5'-AMP.

10. The ATP-supported transport of ^{45}Ca and the Ca^{2+}-induced swelling of mitochondria from spleen, but not from liver, are sensitive to inhibition by Cu^{2+} (40μM). This inhibition is relieved progressively at increased concentrations of Mg^{2+} (0-15mM). Furthermore, Mg^{2+} causes a marked stimulation of ^{45}Ca transport in the absence of added Cu^{2+}; this stimulation is abolished completely upon the removal of the outer membranes of spleen mitochondria, which diminishes the rate of Ca^{2+} uptake by the mitoplasts. In contrast, when succinate is used *in lieu* of ATP, neither Mg^{2+} nor Cu^{2+} influence the transport of Ca^{2+} by spleen mitochondria significantly.

11. Isolated outer membranes from spleen mitochondria, which form tightly-sealed vesicles, are capable of transporting Ca^{2+} in an energy-dependent manner - preferably in the presence of an ATP-regenerating system and Mg^{2+}. Succinate, 3-hydroxybutyrate, Mg^{2+} and P_i do not support Ca^{2+} transport by these vesicles.

12. The translocation of adenine nucleotides by spleen mitochondria is inhibited strongly by Ca^{2+} and to a lesser extent by Mg^{2+}. The modulatory responses of these bivalent cations are not altered upon the removal of the outer membranes.

13. The results are discussed in relation to a proposed functional role of the outer membrane ATPase in spleen mitochondrial transport with particular emphasis on the regulation of Ca^{2+} homeostasis during lymphocyte transformation.

PUBLICATIONS

1. Vijayakumar, E.K. & Weidemann, M.J. (1976) "Location of an Oligomycin-Insensitive and Magnesium Ion-Stimulated Adenosine Triphosphatase in Rat Spleen Mitochondria" Biochem. J 160, 383-393.

2. Vijayakumar, E.K. & Weidemann, M.J. (1977) "Kinetic Properties of a Magnesium Ion-and Calcium Ion-Stimulated Adenosine Triphosphatase from the Outer Membrane Fraction of Rat Spleen Mitochondria" Biochem. J 165, 355-365.

3. Hume, D.A., Vijayakumar, E.K., Schweinberger, F., Russell, L.M. & Weidemann, M.J. (1978) "On the Role of Calcium in the Regulation of Rat Thymocyte Pyruvate Oxidation by Mitogens" Biochem. J (in press).

PAPERS PRESENTED AT CONFERENCES

1. Vijayakumar, E.K., Russell, L.M., Ryall, R.G. & Weidemann, M.J. (1975) "Effect of Divalent Metal Ions on the Oxidation of Pyruvate by Rat Lymphoid Tissue Mitochondria" Proc. Aust. Biochem. Soc. 8, 56.

2. Vijayakumar, E.K. & Weidemann, M.J. (1976) "Kinetic Properties of a Bivalent Metal Ion Stimulated ATPase from the Outer Membrane of Spleen Mitochondria" Proc. Aust. Biochem. Soc. 9, 64.

ABBREVIATIONS

ATPase	adenosine triphosphatase
ATP	adenosine triphosphate
ADP	adenosine diphosphate
AMP	adenosine monophosphate
cAMP	adenosine 3',5'-cyclic phosphate
cm	centimetre
CTP	cytidine triphosphate
conc.	concentration
CCCP	carbonyl cyanide m-chlorophenyl hydrazone
°C	degrees Celsius
c.p.m.	counts per minute
Ci	curie
DNA	deoxyribonucleic acid
DCCD	N,N'-dicyclohexylcarbodiimide
DNP	N,N'-dinitrophenol
d.p.m.	disintegrations per minute
EDTA	ethylenediamine tetra-acetic acid
EGTA	ethanedioxybis (ethylamine) - tetra-acetic acid
E_a	activation energy
FAD	flavin-adenine dinucleotide, oxidized
g	gram
× g	gravitational force
GTP	guanosine triphosphate
GDP	guanosine diphosphate
GMP	guanosine monophosphate
hr	hour(s)
ITP	inosine triphosphate
IDP	inosine diphosphate

K_m	Michaelis constant
K_i	Michaelis inhibitor constant
ℓ	litre(s)
min	minute(s)
M	molar (concentration)
m (prefix)	milli ($10^{-3}\times$)
μ (prefix)	micro ($10^{-6}\times$)
n (prefix)	nano ($10^{-9}\times$)
NTA	nitrilotriacetate
NAD^+	nicotinamide-adenine dinucleotide, oxidized
NADH	nicotinamide-adenine dinucleotide, reduced
$NADP^+$	nicotinamide-adenine dinucleotide phosphate, oxidized
NADPH	nicotinamide-adenine dinucleotide phosphate, reduced
P_i	inorganic phosphate
PP_i	inorganic pyrophosphate
pH	hydrogen ion concentration, minus log of
pKa	dissociation constant, minus log of
p., pp.	page, pages
RNA	ribonucleic acid
RCR	respiratory control ratio
S.E.M.	standard error of estimate of mean value
s	seconds
TMPD	N,N,N',N'-tetramethyl phenylene diamine
UTP	uridine triphosphate
V_{max}	velocity maximum
wt.	weight
(w/v)	weight per volume

CHAPTER 1

INTRODUCTION

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1.1 INTRODUCTION

The work described in this thesis is concerned with the following aspects of the structure and function of mitochondria isolated from rat spleen and thymus:

(a) the activation of pyruvate oxidation by Ca^{2+};

(b) the submitochondrial location and kinetic properties of an oligomycin-insensitive ATPase from spleen that is stimulated by bivalent metal ions; and

(c) aspects of Ca^{2+}-transport by intact mitochondria and by mitochondrial outer membranes that relate to the regulation of the steady-state level of cytoplasmic Ca^{2+}.

To provide an adequate background to these studies, the introductory section contains information about the structure and function of mitochondria and a consideration of the properties of the ATPases located in the inner mitochondrial membrane, the plasma membrane and the membrane of the sarcoplasmic reticulum. As well, information about the control of cellular Ca^{2+} and its possible role in cell activation is discussed.

1.2 STRUCTURE OF MITOCHONDRIA

1.2.1 Introduction

The name "mitochondrion" is derived from the Greek *"mitos"* meaning a thread, and *"chondros"*, a grain. In the mid to late 19th century, cytologists observed granular elements and filamentous structures in the cytoplasm of many different types of cells. In 1946, Claude attempted to isolate and separate "large granules" (mitochondria) and "small granules" (microsomes) from liver homogenates by differential centrifugation. The "large granule" fraction contained mitochondria that were damaged and

contaminated with impurities. In 1948 Hogeboom, Hotchkiss and Schneider, by dispersing rat liver in hypertonic sucrose medium (0.88M), succeeded in isolating mitochondria that were intact and relatively uncontaminated with material from other cell fractions. Based on this method, Kennedy and Lehninger (1949) demonstrated that the degree of intactness of the mitochondria was related to the osmolarity of the isolation medium. Only the mitochondrial fraction isolated in iso-osmotic sucrose medium (0.25M), and not the nuclear or the soluble fraction, was capable of oxidizing tricarboxylic acid cycle intermediates. In 1951, Green and his co-workers confirmed this finding with the isolation of a "cyclophorase" preparation from kidney and liver homogenates that, with hindsight, appears to have been a mitochondrial fraction. Methods are now available for isolating mitochondria from most types of eucaryotic cells including tumour cells (Wu and Sauer, 1967; Thorne and Bygrave, 1974), insect flight muscle (van Den Berg, 1967; Wohlrab, 1975; Bygrave *et al.*, 1975), plant tissue (Bonner, 1967; Day and Wiskich, 1975) and micro-organisms (Mattoon and Balcavage, 1967).

1.2.2 Organization of the membranes of mitochondria

The principal structural elements of mitochondria consist of a smooth outer membrane and an involuted inner membrane. The inner membrane is a closed sac, surrounded by the outer membrane, so that the whole structure can be pictured as a sac within a sac. The inner membrane is folded and involuted into structures known as *cristae mitochondrioles* (Palade, 1952) which greatly increase its surface area and provide higher local concentrations of the enzymes responsible for electron transport and oxidative phosphorylation.

The other mitochondrial components are the *matrix space* (the inner compartment) which is bounded by the inner membrane, and the *periplasmic* (intermembrane) *space* which is situated between the two membranes. The

matrix and its boundary membrane constitute the "mitochondrion" in a functional sense, as this compartment is a closed system containing the enzymic components responsible for substrate oxidation, respiration and energy conservation.

1.2.3. Size, shape, area and number of mitochondria in the cell

In the cells of liver and kidney, mitochondria are elongated structures about 3μ long and 0.5 to 1.0μ in diameter. In other cell types (e.g., pancreas) the mitochondria may be extremely elongated rods or filaments with a length of up to 10μ. The surface area of the outer membrane of a typical liver mitochondrion is approximately $13\mu^2$ or about one third of the surface area of the inner membrane. It is estimated that the total surface area of the two mitochondrial membranes in a typical liver cell is more than ten times that of the plasma membrane (Munn, 1976).

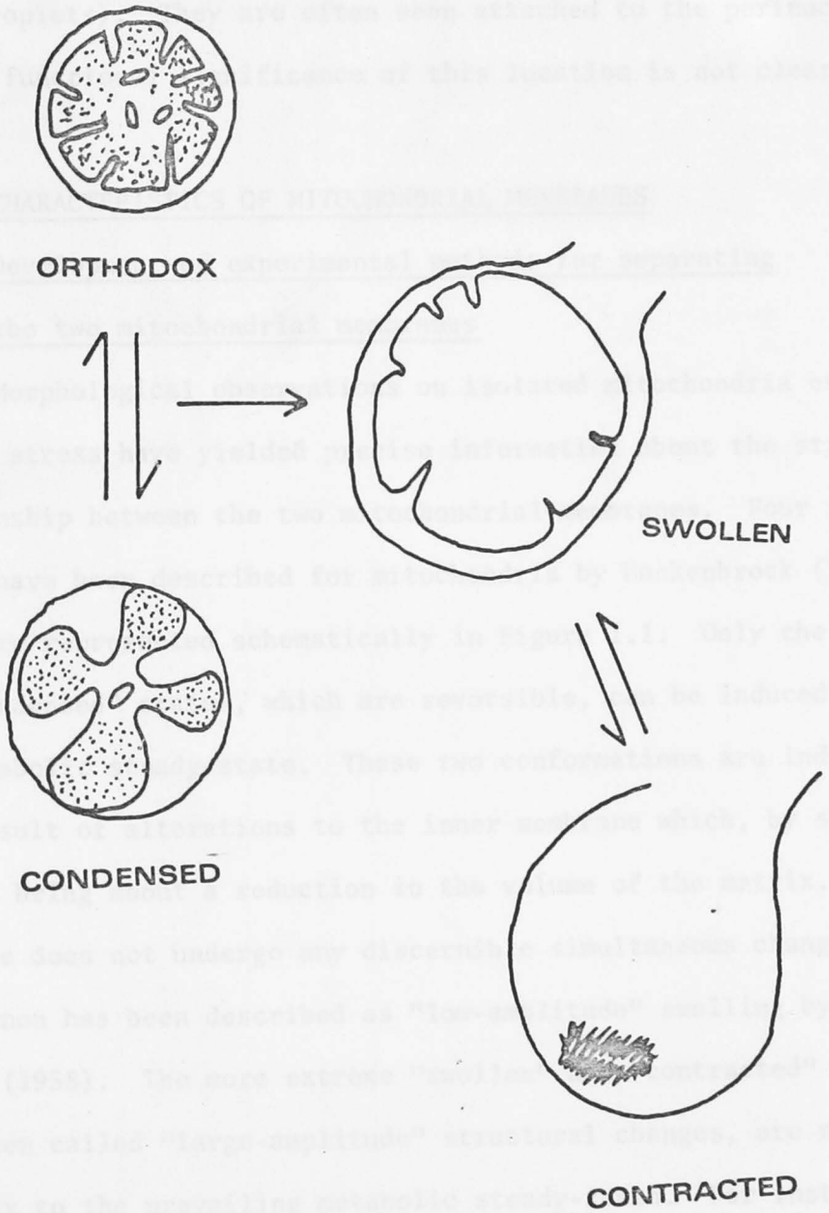
Rat hepatocytes have been reported to contain 500-2000 mitochondria per cell, whilst renal tubule cells - with a somewhat higher respiratory capacity - contain only 300. Lymphoid tissues, which are dependent on a high rate of glycolysis for their energy supply (Suter and Weidemann, 1975; 1976), contain only 20-50 mitochondria per typical small lymphocyte (Munn, 1976). Some giant amoebae contain over half a million mitochondria per cell.

1.2.4 Intracellular location of mitochondria

The location and orientation of mitochondria within the cell varies with the cell type. In pancreatic acinar cells, for instance, the mitochondria are oriented concentrically, presumably to assist secretory process. Mitochondria associated intimately with the rough-surfaced endoplasmic reticulum in other cells may provide ATP for amino acid activation and protein synthesis by the ribosomes. In skeletal muscle, the mitochondria are arranged so that they are in close physical contact with the contractile

Figure 1.1 Conformational states of mitochondria

(from Ernster and Kuylenstierna, 1968)


ORTHODOX
SWOLLEN
CONDENSED
CONTRACTED

elements of the myofibrils (Novikoff, 1961).. In many cell types the mitochondria appear to be located near an abundant supply of substrate (e.g., lipid droplets). They are often seen attached to the perinuclear membrane, but the functional significance of this location is not clear.

1.3 CHARACTERISTICS OF MITOCHONDRIAL MEMBRANES

1.3.1 Development of experimental methods for separating the two mitochondrial membranes

Morphological observations on isolated mitochondria exposed to osmotic stress have yielded precise information about the structural relationship between the two mitochondrial membranes. Four structural states have been described for mitochondria by Hackenbrock (1966) and these are represented schematically in Figure 1.1. Only the "orthodox" and "condensed" states, which are reversible, can be induced by altering the metabolic steady-state. These two conformations are induced primarily as a result of alterations to the inner membrane which, by shrinking, is able to bring about a reduction in the volume of the matrix. The outer membrane does not undergo any discernible simultaneous change. This phenomenon has been described as "low-amplitude" swelling by Chance and Packer (1958). The more extreme "swollen" and "contracted" states, which have been called "large-amplitude" structural changes, are not related directly to the prevailing metabolic steady-state. For instance, exposure of liver mitochondria to a hypotonic medium containing permeable cations and anions (e.g., K^+, Ca^{2+}, P_i) (Chappell, 1968) or to a medium containing short-chain fatty acids and thyroxine (Lehninger, 1962) leads to swelling which may be reversed by the subsequent exposure of swollen mitochondria either to hypertonic medium or a variety of other agents (e.g., ATP, Mg^{2+} or bovine serum albumin). During controlled swelling the inner membrane is largely unfolded into a reversible conformation that can be restored

Table 1.1

Cholesterol, phospholipid and protein contents of mitochondrial membranes from liver and heart

CONTENTS	PIG LIVER MITOCHONDRIA (Parsons & Yano, 1967)			PIG HEART MITOCHONDRIA (Comte *et al.*, 1976)		
	Whole	Outer Membrane	Inner Membrane	Whole	Outer Membrane	Inner Membrane
Phospholipids (mg/mg protein)	0.159	0.878	0.301	0.242	0.614	0.372
Cholesterol (µg/mg protein)	2.28	30.1	5.06	4.35	9.4	1.7
Protein (percentage)	100	4.5	76.0	100	6.4	60.4

to its normal state by contraction. During this process the outer membrane is often so distended that it becomes detached from the inner membrane (Parson, 1965; Munn and Blair, 1967). These properties have been important for the design of methods for separating the two membranes.

A method for separating the "outer" and "inner" membranes was described first by Parson and his co-workers (Parson and Williams, 1967). Only the outer membrane ruptures on swelling in hypotonic P_i-buffer (Wlodawer *et al.*, 1966) while the inner membrane unfolds without breaking. The two membrane fractions can then be purified further by differential centrifugation through a suitable gradient (e.g., sucrose). Schnaitman *et al.* (1967) have used distilled water instead of P_i-buffer as a hypotonic medium to swell mitochondria. The "ghost" mitochondria obtained by this method, containing mostly inner membranes (Caplan and Greenawalt, 1966), were separated from the fragmented outer membranes by differential centrifugation through a sucrose gradient.

All of these early methods yielded sub-fractions that were impure due to inadequate separation. Sottocasa *et al.* (1967a) developed a modified method in which mitochondria swollen in P_i-buffer were treated with hypertonic sucrose medium containing ATP and Mg^{2+} to contract the inner membranes selectively and to facilitate the detachment of the broken outer membranes (Sottocasa *et al.*, 1967b). The sample was then subjected to controlled sonic oscillations to cause maximum rupture of the outer membrane; this gave, after differential centrifugation, a high yield of relatively pure membrane particles (Sottocasa *et al.*, 1967c; Hayashi and Capaldi, 1972). Levy *et al.* (1967) introduced a new technique, later modified by Schnaitman and Greenawalt (1968), based on the observation that digitonin at low concentration can specifically esterify the cholesterol-rich outer membrane (see Table 1.1) and detach it from the rest of the mitochondrion (Levy and Sauner, 1967; Schnaitman *et al.*,

Table 1.2

Methods for the separation of the outer and inner mitochondrial membranes

(from Racker, 1970)

Abbreviations: OM - outer membrane; IS - inter-membrane space; IM - inner membrane; Supnt - supernatant; conc - concentration; M - Matrix; diff - differential; dens. gr. - density gradient; centr - centrifugation

REFERENCE	TISSUE	RATIONALE	TREATMENT	MEANS OF SEPARATION
Parsons *et al.* (1966; 1967)	Rat Liver & Guinea Pig liver	Rupture of OM by swelling	Hypotonic P_i	Diff. and dens. gr. centr
Sottocasa *et al.* (1967a, b, c)	Rat liver	Rupture & detachment of OM by swelling & contraction followed by mechanical fragmentation of OM	Hypotonic P_i, followed by hypertonic sucrose + ATP + Mg^{2+} & sonication	Dens. gr. centr
Schnaitman *et al.* (1967)	Rat liver	Rupture of OM by swelling. Binding of OM-cholesterol by digitonin	Distilled water. Low conc of digitonin	Diff gr centr. Diff centr

Table 1.2 (contd)

REFERENCE	TISSUE	RATIONALE	TREATMENT	MEANS OF SEPARATION
Levy *et al.* (1967)	Rat liver	Binding of OM-cholesterol by digitonin, followed by mechanical fragmentation	Low conc of digitonin, sonication	Diff and dens. gr. centr
Green *et al.* (1966)	Beef heart	Separation of particulate subfractions with and without respiratory chain enzyme components	Sonication in the presence of cholate	Diff centr
Allman *et al.* (1966)	Rat liver	Detachment of OM by swelling and contraction	Oleate followed by ATP + Mg^{2+} + serum albumin	Diff centr

1967). The modified technique gave a well-preserved, non-leaky inner membrane plus matrix ("mitoplast") fraction after differential centrifugation that exhibited good respiratory control.

Thus, the separation of the inner and outer membranes of most mammalian mitochondria is a relatively easy process, employing either high-amplitude swelling in the presence of P_i, or digitonin fractionation. With mitochondria from certain tissues, such as heart, the problem is more difficult, because the outer membranes have a very small surface area compared with the number of cristae. Furthermore, the cholesterol content of pig heart membranes (Table 1.1) appears to be low compared with that of liver mitochondria (Graham and Green, 1970; Colbeau *et al.*, 1971). This difference makes it difficult to use digitonin fractionation to isolate the outer membranes effectively (Scholte *et al.*, 1973; Comte *et al.*, 1976). Several attempts have been made to separate the two mitochondrial membranes from beef (Green *et al.*, 1966; Hayashi and Capaldi, 1972) and guinea-pig heart (Jacobus and Lehninger, 1973; Scholte *et al.*, 1973). Comte *et al.* (1976) have described a satisfactory method, based on the principle of controlled osmotic swelling and membrane disruption, for isolating relatively pure outer membranes from pig heart mitochondria.

Among various methods that are summarised in Table 1.2, digitonin-fractionation (Schnaitman *et al.*, 1967) is still considered to be the method of choice in most cases as studies from several laboratories on the distribution of intra-mitochondrial enzymes and metabolites have shown remarkable agreement where this method has been used.

1.3.2 Physical characteristics of the inner and outer membranes of mitochondria

The different buoyant densities of outer (1.13) and inner (1.21) membranes of liver mitochondria (Parsons *et al.*, 1966), can be correlated with their relative content of phospholipid and protein (Table 1.1). The inner membrane, which is 50-70 Å in thickness, has regularly-shaped, mushroom-like projections of 75-80 Å in diameter attached to its inner surface (Fernandéz-Moran, 1962). Each of these projections has ATP-synthase activity and an electron transport system associated with it. Parson (1965) has identified projecting subunits on the outer membranes of liver mitochondria also, but they differ from those of the inner membrane in size (60 Å), shape (hollow cylindrical) and regularity. However, the enzymic composition or the functional significance of these projections is not known.

As noted previously, the osmotic behaviour of the two membranes is remarkably different: only the inner membrane unfolds or refolds in response to osmotic changes, whereas the outer membrane becomes distended and often ruptures. This difference in placticity would tend to suggest that contractile proteins (Ohnishi and Ohnishi, 1962) might be associated with the inner but not the outer membranes.

Studies on mitochondrial permeability have shown that the inner membrane is impermeable to most substances unless specific carriers are available for them or unless the molecules in question are neutral and of molecular weight smaller than 150 daltons (Klingenberg, 1972). On the other hand, the outer membrane of liver mitochondria appears to be permeable to a wide range of substances, irrespective of charge, of molecular weight up to about 10,000 daltons. The outer membranes of mitochondria from non-hepatic tissues may have quite different permeability properties to those of liver (Gmaj *et al.*, 1974) and this possibility will be discussed in later chapters.

Table 1.3

Location of enzymes in the outer membranes of mitochondria*

ENZYMES	TISSUE STUDIED	REFERENCES
Monoamine oxidase	Rat liver, Guinea-pig heart, beef heart	Sottocasa *et al.* (1967a); Schnaitman *et al.* (1967); Maisterrena *et al.* 1974); Comte *et al.* (1976).
Kynurenine hydroxylase	Rat liver, Neurospora	Cassady & Wagner (1971); Day & Wiskich (1975).
Rotenone-insensitive $NADH_2$-cyt b_5 oxidoreductase	Rat liver, beef heart	Sottocasa *et al.* (1967a); Douce *et al.* (1968); Comte *et al.* (1976)
Xylitol dehydrogenase (NAD-specific)	Rat liver	Arsenis *et al.* (1968)
Acetyl-CoA carnitine O-acyl-transferase	Rat liver	Higgins & Barrnett (1970)
Acyl-CoA L-Glycerol 3-phosphate O-acyl transferase	Rat liver	Stoffel & Scheifer (1968)
Acyl-CoA lysolecithin O-acyl-transferase	Rat liver	" " " "
Acyl-CoA lysophosphatidic acid O-acyl transferase	Rat, calf and human liver	" " " "
Acyl-CoA ligase	Rat liver	Farstad *et al.* (1967); Pande & Meade (1968)

Table 1.3 (contd)

ENZYMES	TISSUE STUDIED	REFERENCES
L-α-phosphatidate phosphohydrolase	Rat liver	Stoffel & Scheifer (1968); Sedgwick & Hübscher (1965)
Phospholipase A_{II}	Rat liver	Vignais *et al.* (1966)
Choline phosphotransferase	Rat liver	Stoffel & Scheifer (1968)
Fatty acid elongation system	Rat & ox liver	Colli *et al.* (1968)
ATP: D-hexokinase-6 phosphotransferase	Guinea-pig: brain, liver & intestine and numerous other sources	Kropp & Wilson (1970); Mayar & Hubscher (1971); Rowsell *et al.* (1972)
Bicarbonate-stimulated ATPase	Rat liver	Grisolia & Mendelsen (1974)
Ca^{2+}- and Mg^{2+}- stimulated ATPase	Rat kidney - cortex	Gmaj *et al.* (1974)

*Data compiled from: (1) Munn (1976) and

(2) Racker (1970)

1.4 ENZYMES AND STRUCTURAL COMPONENTS OF THE MEMBRANES OF MITOCHONDRIA

1.4.1 The mitochondrial outer membrane

The outer membrane has not been as well characterised as the inner membrane of mammalian mitochondria. The mitochondrial outer membrane from beef heart contains 40 percent by weight phospholipid and 60 percent protein (Smoly *et al.*, 1971; Hayashi and Capaldi, 1972). The sialic acid content is far higher than that of the inner membrane and most of it is associated with glycolipid (Hayashi and Capaldi, 1972; Melnick *et al.*, 1973). A glycoprotein with a molecular weight of 93,000 daltons has been identified in the outer membrane of rat liver mitochondria (Melnick *et al.*, 1973). The outer membranes of most mammalian mitochondria, when solubilized in sodium dodecyl sulphate (S.D.S.), contain about 12-14 different polypeptides that can be separated by polyacrylamide gel electrophoresis (Schnaitman, 1969; Smoly *et al.*, 1971; Hayashi and Capaldi, 1972; Melnick *et al.*, 1973; Comte *et al.*, 1976). The molecular weights of these proteins have been estimated to be in the range of 12,000-220,000 daltons (Capaldi, 1977).

Three different enzyme activities have been identified positively as associated with the outer membrane fraction. Two of them, kynurenine hydroxylase and rotenone-insensitive NADH-cytochrome reductase, have not been studied in detail. In contrast, monoamine oxidase is well characterized. Table 1.3 lists these enzymes and others for which an outer membrane localization has been suggested in a variety of mammalian tissues.

1.4.2 The mitochondrial inner membrane

The composition of the mitochondrial inner membrane is better defined than that of all other mammalian membranes. It differs markedly from the mitochondrial outer membranes in having a high content of cardiolipin and a low level of cholesterol (see Table 1.1). Whilst a

Table 1.4

Enzyme components of inner membrane of mitochondria

ENZYMES	REFERENCES
Complex I - (2 major and 5 minor polypeptides) NADH-coenzyme Q reductase (NADH dehydrogenase with its five different non-haem iron centres in the complex; NADPH dehydrogenase)	Ohnishi *et al.* (1972); Orme-Johnson *et al.* (1971); Baugh & King (1972); Hatefi (1973).
Complex II - (3 major and 4 minor polypeptides) Succinic-coenzyme Q reductase (succinate dehydrogenase with its flavin and non-haem iron protein: cyt-b and another non-haem iron protein)	Davies & Hatefi (1971); Righetti & Cerletti (1971); Davies *et al.* (1972); Baginsky & Hatefi (1968).
Complex III - Reduced coenzyme Q - cytochrome c reductase (cyt-c; cyt-b: two molecules b_k & b_t; a non-haem iron protein)	Rieske *et al.* (1964); Yu *et al.* (1972); Chance *et al.* (1970); Davies *et al.* (1972); Albrecht & Slater (1971).
Complex IV - Cytochrome c oxidase (7 different proteins and 2 haems and 2 copper moieties associated with one or more polypeptides)	Capaldi & Hayashi (1972); Kubayoma *et al.* (1972); Wainio *et al.* (1973); Komai & Kapaldi (1973).
Complex V - ATP-synthase or ATPase	See text

Table 1.4 (contd)

Electron transfer components	
cytochrome c	Dickerson *et al.* (1971); Smith *et al.* (1973)
L-α Glycerophosphate oxidase	Ringler & Singer (1958); Ringler (1961)
3-hydroxybutyrate dehydrogenase	Hexter & Goldman (1973); Menzel & Hammes (1973)
Choline dehydrogenase	Singer (1963)
Coenzyme Q	Morton *et al.* (1961)
Transport components (carrier proteins)	
Adenine nucleotide transporter	Weidemann *et al.* (1970)
Ca^{2+} transporter	Lehninger (1973); Carofoli (1973)
Na^{+}, K^{+} and Mg^{2+} ionophore	Blondin (1974)
P_i transporter	McGivan & Klingenberg (1971)
Succinate, Malate, P_i, glutamate, aspartate... transporter systems	Quagliariello *et al.* (1969); Palmieri *et al.* (1971)
Pyruvate and α-oxoglutarate	Halestrap & Denton (1974); Halestrap (1975)
Citrulline and ornithine	Gramble & Lehninger (1973)

Data on Complexes I to V is taken from Capaldi (1977); and the other data is selected from the literature as indicated.

large proportion of this lipid bilayer is undoubtedly "fluid" at physiological temperatures (Capaldi and Green, 1972), a significant proportion is immobilised by association with proteins (like cytochrome oxidase) that are intrinsic inner membrane components (Jost *et al.*, 1973).

A large number of proteins in the inner membrane (with many different enzymic activities) are associated with haem, flavin, copper and non-haem iron moieties (see Table 1.4). The majority of the protein components of the inner membrane are associated into five complexes: four electron-transfer complexes (complexes I - IV; see Table 1.4) and an ATP hydrolysing and synthesizing complex (Capaldi, 1977). Apart from the enzymes that are listed in Table 1.4, the inner membrane contains a number of other activities such as steroid 11-β-hydroxylase (Sweat, 1951; Yago and Ichii, 1969) pyrophosphate phosphohydrolase (Nordlie and Lardy, 1961; Schick and Butler, 1969) and protohaem-ferrolyase (Jones and Jones, 1968).

The electron transfer complexes, the ATP-synthase and the ion translocating components (see Table 1.4) are all functionally integrated. During electron transfer, NADH at complex I or succinate at complex II is oxidized; the electrons separated as a result of these oxidation reactions are transferred through complex III to complex IV, where they combine with protons and molecular oxygen. The sequence of events is summarized below:

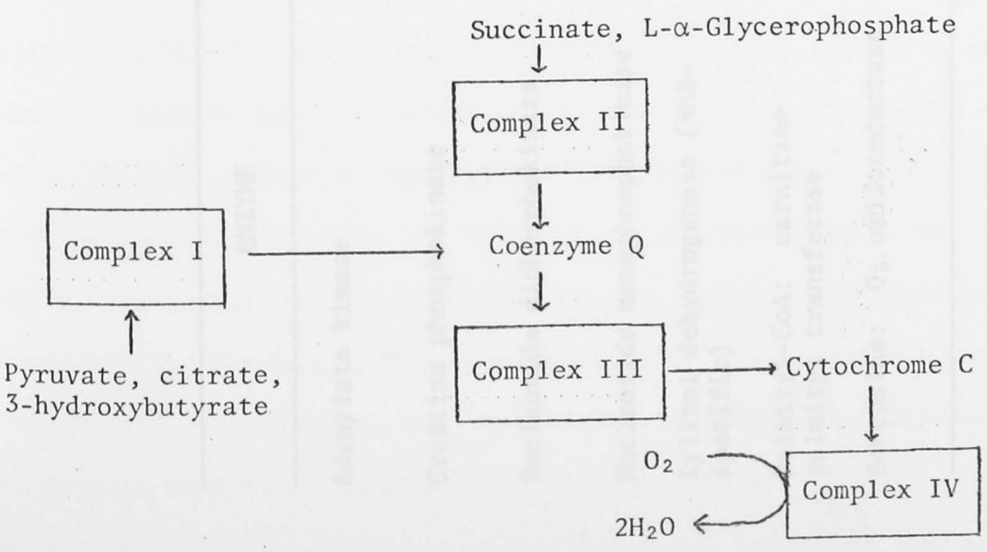


Table 1.5

Enzymes of mitochondrial intermembrane space

(from Racker, 1970; and Munn, 1976)

ENZYME	SOURCE	REFERENCE
Adenylate kinase	Rat liver, kidney-cortex	Sottocasa *et al.* (1967a); Schnaitman and Pedersen (1968)
Creatine phosphokinase	Rat brain, rat heart, guinea-pig brain	Jacobs *et al.* (1964); Jacobus and Lehninger (1973)
Nucleotide diphosphokinase	Rat liver, rat kidney-cortex	Lima *et al.* (1968); Schnaitman and Greenawalt (1968)
Nucleoside monophosphokinase	Rat liver	Lima *et al.* (1968)
Xylitol dehydrogenase (NAD-specific)	Rat liver	Arsenis *et al.* (1968)
Palmityl-CoA: carnitine palmityl transferase	Rat, calf and human liver	Yates & Garland (1966)
Sarcosine: O_2 oxidoreductase	Rat liver	Hoskins & Mackenzie (1961)

Energy is generated by electron transfer through complexes I, III and IV. This energy can be utilized by the ATP-synthase complex for ATP synthesis or it can be used by ion-translocating components. The system can also work in the reverse direction, resulting in the ATP-synthase catalyzed hydrolysis of ATP. The energy released can be used to reverse electron transport (Lee and Ernster, 1966) or to drive ion-transport (Lehninger *et al.*, 1967).

1.4.3 ATP-synthase (ATPase) of the mitochondrial inner membrane

Of the enzymes listed in Tables 1.4 and 1.5, special emphasis is given to the properties of the ATP-synthase of the mitochondrial inner membrane, as an ATP-hydrolysing enzyme from the outer membrane of (spleen) mitochondria that has some similar properties is described in this thesis. ATP synthesis and hydrolysis are the function of a single complex variously called the reversible ATPase (Racker, 1972), oligomycin-sensitive ATPase (Tzagoloff *et al.*, 1968) or $C_oF_oF_1$ (Kagawa and Racker, 1966), but in this Chapter it is called "ATP-synthase" or ATPase. The ATPase activity of the mitochondrial inner membrane is of great interest to biochemists because of its dual role in energy-dependent ion transport and oxidative phosphorylation. The same enzyme has been found in almost all animal cell mitochondria as well as in chloroplasts and micro-organisms. Because of the functional homologies shared by these enzymes of different origin, especially in the catalysis of the terminal reactions of oxidative phosphorylation, many workers have focussed attention on their common catalytic and structural properties. The mitochondrial ATPase bears a striking resemblance to the chloroplast and bacterial enzymes, especially with respect to its molecular weight, amino acid composition (Schnebli and Abrams, 1970), appearance under the electron microscope (Kagawa and Racker, 1966) and sensitivity to inhibitors such as oligomycin and DCCD (Catterall and Pedersen, 1971).

Table 1.6

Molecular structure of ATPase or "ATP-synthase"
[from Sone *et al.* (1975); and
Kagawa *et al.* (1976)]

Molecular structure of F_1

SUBUNITS		
Number	Molecular Weight	Ratios
1 (α)	56,000	3
2 (β)	53,000	3
3 (γ)	32,000	1
5 (δ)	15,500	1
7 (δ^1)	11,000	1

Molecular structure of F_o:

SUBUNITS		
Number	Molecular Weight	Ratios
4 (γ^1)	19,000	1
6 (ε)	13,500	2
8 (ε^1)	5,400	5

1.4.3.1 Components of the ATPase

In its intact form, the complete ATPase complex present in the mitochondrial inner membrane is believed to consist of four functional components: a head-piece - called F_1 - which is capable of hydrolyzing ATP (Pullman *et al.*, 1960); a membrane component which is capable of directing the flow of protons to F_1 during oxidative phosphorylation (Mitchell, 1973, 1974; Kagawa *et al.*, 1976; Okamoto *et al.*, 1977); a basic peptide or "stalk" which binds F_1 to the membrane component and confers oligomycin sensitivity on it (Beechey *et al.*, 1967; MacLennan and Tzagoloff, 1968); and an inhibitor peptide which prevents the hydrolysis of ATP during oxidative phosphorylation (Pullman and Monroy, 1963; Serrano *et al.*, 1976).

Although the complete oligomycin-sensitive ATPase complex was not purified before the 1970's, an ATPase identified with coupling factor I (F_1) of oxidative phosphorylation was purified earlier from beef heart mitochondria (Pullman *et al.*, 1960; Penefsky *et al.*, 1960). Later, similar ATPases capable of oxidative phosphorylation, photophosphorylation and translocation of ions were isolated (Senior, 1973; Pedersen, 1975). These ATPases all have similar molecular weights of 3.5×10^5 daltons (Kagawa and Racker, 1966; Senior, 1973; Pedersen, 1975) and can be resolved into five similar subunit polypeptides, three large (α,β and γ) and two small (δ and ε). The molecular structure of the subunits of F_1 is given in Table 1.6 (Kagawa *et al.*, 1976). F_1 is not inhibited by inhibitors of energy-transfer (e.g., oligomycin) which inhibit the intact membrane-bound ATPase. Kagawa and Racker (1966) identified a group of hydrophobic, intrinsic membrane-bound proteins called F_o which confer sensitivity to various inhibitors on F_1. Subsequently, the individual components of F_o have been purified; its complete molecular structure (Kagawa *et al.*, 1976) is shown in Table 1.6.

Table 1.7

Catalytic properties of mitochondrial ATPase

(from Pedersen, 1975)

PROPERTY	GENERAL COMMENTS
Activity in the absence of metal ions	Low, but significant rates of ATP hydrolysis are reported
Specificity of metal ions	Mg^{2+}, Co^{2+}, Mn^{2+}, Ca^{2+} and Fe^{2+} support hydrolysis. The specificity of cations depends on both K^+ and, ATP concentrations
Specificity for NTPs (Nucleoside triphosphate)	Membrane bond: ATP>ITP>GTP>UTP>CTP. For purified F_1: ATP, ITP and GTP hydrolytic rates are equal (almost). UTP and CTP are hydrolysed very slowly.
K_m for ATP	Membrane-bound: K_m = 0.1 - 0.3mM; For F_1: K_m = 0.75 - 1.25mM.
Activators (anions)	Bicarbonate, P_i, bisulfate, borate, maleate, terephthalate, dichromate, etc.; 2-fold activation.
Inhibitors:	
(i) ADP	Membrane-bound: K_i values = 3μM to 4mM F_1: K_i = 30μM to 300μM The reason for apparent discrepancies is not known.
(ii) ATP-analogues (AMP-PNP)	competitive inhibition; ATPase but not ox.phos inhibition
(iii) Inhibitors of oxidative phosphorylation	Membrane-bound: Oligomycin, DCCD, rutamycin, aurovertin-B, tri-n-butyl tin, venturicidin, quercetin, spegazzine, and ATPase antibody. F_1: aurovertin, Azide, Quercetin, spegazzine, ATPase antibody.

Although purified preparations of oligomycin-insensitive ATPase have been useful in elucidating the molecular structure of the enzyme, experiments aimed at reconstituting and resolving the mechanism of oxidative phosphorylation have been quite limited owing to difficulties encountered in purifying the oligomycin-sensitive ATPase complex. Several preparations of oligomycin-sensitive ATPases from yeast (Tzagoloff and Meager, 1971; Ryrie, 1975), beef heart (Swanljung *et al.*, 1973; Sadler *et al.*, 1974) and thermophilic bacteria (Yashida *et al.*, 1975) have been described, which have enabled the workers concerned to study the functional properties of each individual component in relation to that of the ATPase complex as a whole. Some important properties of the enzyme are summarized in Table 1.7.

1.4.3.2 Functional properties

According to the chemiosmotic hypothesis of oxidative phosphorylation, ATP can be synthesised from ADP and P_i when the reaction is driven by a proton motive force applied across the inner mitochondrial membrane (Mitchell, 1966). It was suggested that F_o serves as a proton "conductor" through the membrane, and that the energy transfer inhibitors (e.g., oligomycin) exert their effects by blocking proton conduction through F_o (Mitchell, 1966). In fact, crude F_o vesicles that were constructed in the presence of F_1 showed inhibitor-sensitive oxidative phosphorylation (Racker and Kandrach, 1973), proton translocation (Kagawa *et al.*, 1973), and ^{32}P-ATP exchange (Kagawa and Racker, 1971). Moreover, passive proton translocation in F_1-depleted submitochondrial particles or vesicles that still contained crude F_o was shown to be inhibited by energy-transfer inhibitors (Racker, 1972; Schipakin *et al.*, 1976). However, such F_o preparations contained considerable impurities, including electron transport chain components (Kagawa and Racker, 1971; Ragan and Racker, 1973), and were not stable enough for quantitative analysis of proton conduction

through F_o under the desired conditions.

More recently, a highly stable ATPase complex (T F_oF_1) has been purified from the membranes of a thermophilic bacteria (Kagawa, 1976) and a stable F_o portion (TF_o) obtained from it (Yashida *et al.*, 1975; Sone *et al.*, 1975). Using reconstituted vesicles from TF_o and phospholipids, Kagawa and his co-workers were able to demonstrate conduction of protons across the membrane driven by a potential difference (created with a gradient of K^+ ions by addition of valinomycin) (Okamoto *et al.*, 1977). The ^{32}P-ATP exchange activity of the TF_o vesicles, which represents the physiological activity of the proton-translocating ATPase complex, was demonstrated in the presence of TF_1 in the same study (Okamoto *et al.*, 1977). This work provides biochemical evidence to support the proposal that the reaction catalyzed by the ATPase (F_oF_1) is in reversible equilibrium with the "energised state" of the membrane (Racker, 1972); when this state is generated by an ion gradient or electron transport, ATP is synthesized; conversely, the hydrolysis of ATP can generate the "energised-state" and so drive active ion transport and reverse electron transport (Serrano *et al.*, 1976).

1.5 ATPases OF THE SARCOPLASMIC RETICULUM AND PLASMA MEMBRANE

1.5.1 Introduction

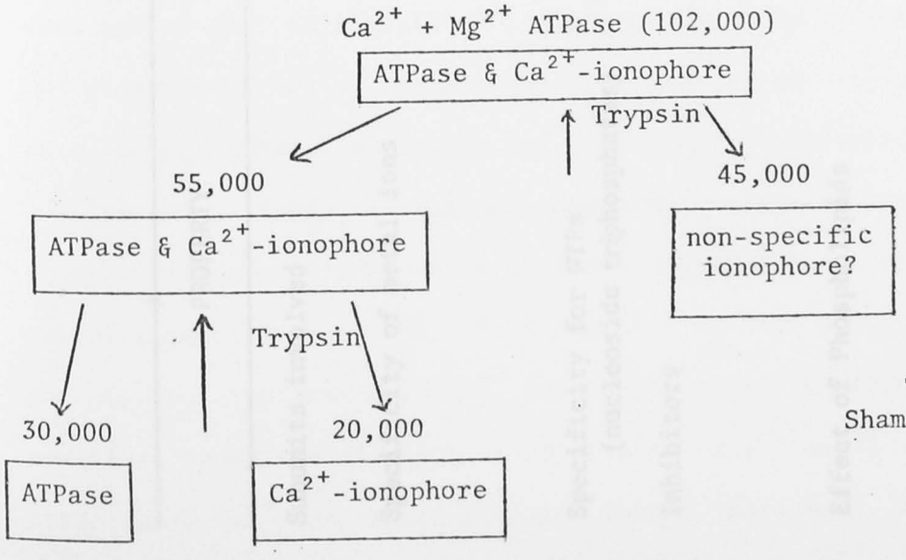
It is clear that the transport function of the mitochondrial inner membrane is separable from the system that hydrolyses ATP, since Ca^{2+} transport, for example, can be supported by energy derived from electron transport (Chance, 1965). In contrast, when Ca^{2+} is transported by the sarcoplasmic reticulum or when Na^+ and K^+ are transported across the plasma membrane, it is conceivable that the ATPase and the ion-transporting enzyme are one and the same protein (Skou, 1965), since transport depends exclusively on ATP hydrolysis. Many approaches have been made towards identification of enzymes, enzyme subunits and fragments of

enzymes which possess the capacity to transport ions across a lipid bilayer (Shamoo and MacLennan, 1974; Serrano *et al.*, 1976; Okamoto *et al.*, 1977). The purified sarcoplasmic reticulum is an excellent system for studying the resolution and reconstitution of transport, as the membrane is specialised for one single transport function (Ca^{2+}-transport) (MacLennan, 1970; Makinose and Hasselbach, 1971) and there appears to be no other enzyme activity present apart from a powerful ATPase. Thus, consideration of such a system, with its ATPase activity, may provide guidelines for distinguishing and defining a functional role for the outer membrane-bound ATPase of spleen mitochondria that is described in this thesis.

1.5.2 ATPase of sarcoplasmic reticulum

1.5.2.1 Components

In contrast to the mitochondrial ATP synthase, the sarcoplasmic reticulum ATPase consists of only a single subunit with a molecular weight of 100,000 daltons (Hasselbach, 1964; Martonosi, 1968; MacLennan, 1970). Controlled tryptic cleavage of this enzyme gives two fragments with molecular weights of 55,000 and 45,000 daltons (Migala *et al.*, 1973; Stewart *et al.*, 1976). The site of ATP hydrolysis, cleaved from the 100,000 dalton fragment, is localized in the 55,000 dalton fragment;



Taken from Shamoo & Goldstein (1977)

Table 1.8

Properties of sarcoplasmic reticulum ATPase

PROPERTY	GENERAL COMMENTS	REFERENCES
Subunits involved	only one: 100,000 Daltons	Hasselbach (1964); MacLennan (1970); Martonosi (1971)
Specificity of metal ions	requires Mg^{2+} and is stimulated by Ca^{2+}. Ba^{2+}, Cd^{2+} cannot replace Ca^{2+}. Mg^{2+} can be replaced by Mn^{2+} or Co^{2+} but not by Sr^{2+}, Zn^{2+} etc.	MacLennan (1970); Makinose & Hasselbach (1971)
Specificity for NTPs (nucleoside triphosphates)	Order of specificity: ATP>GTP>ITP>UTP>CTP	MacLennan (1970); Barlogie *et al.* (1971)
Inhibitors	Specific for mersalyl ($K_i \sim 0.5\mu M$); EGTA, Caffeine, ouabain, rutamycin, etc. have no effect	MacLennan (1970)
Effect of Phospholipids	Removal of phospholipids (e.g., cholate extraction) results in complete loss of activity; phospholipase-C digestion inhibits 50% activity even if supplemented with phospholipids.	Martonosi *et al.* (1968); Hasselbach (1974)

active site analysis shows that this fragment is normally located on the outer surface of the sarcoplasmic reticulum membrane (Stewart *et al.*, 1976) and contains a selective ionophoretic activity for Ca^{2+} (Thorley-Lawson and Green, 1973). Further cleavage of the 55,000 dalton fragment gives rise to a 30,000 and a 20,000 dalton fragment (Stewart *et al.*, 1976); the Ca^{2+}-dependent ionophoretic activity is located in the 20,000 dalton fragment and the ATP hydrolytic site in the 30,000 dalton fragment. These two sites, however, appear to be in close proximity in the native enzyme since both fragments originate from the 55,000 dalton fragment. It is suggested that the role of the 45,000 dalton fragment may be to form a large non-specific channel across the membrane (Shamoo and Goldstein, 1977).

1.5.2.2 Properties and function

Two ATP hydrolysing activities have been resolved in isolated sarcoplasmic reticulum membranes; a Ca^{2+}-dependent and a Ca^{2+}-independent ATPase (Hasselbach and Makinose, 1961; 1963). In the absence of Ca^{2+} ions, ATP is hydrolysed slowly by an enzyme called "basic ATPase" that is moderately activated by Mg^{2+} ions and hydrolyses only ATP and ITP (Hasselbach, 1974). The activity is not inhibited by ADP and is insensitive to thiol-reagents. The Ca^{2+}-dependent ATPase is a highly active form of the same enzyme that is stimulated by Ca^{2+} at low concentration (0.1μM) in the presence of Mg^{2+}. It is capable of hydrolysing all nucleoside tri-phosphates, carbamyl phosphate and p-nitrophenyl phosphate (Friedman and Makinose, 1970). If the Ca^{2+}-dependent enzyme is purified, it can be phosphorylated with ATP, which causes a commensurate increase in the ATPase activity (MacLennan, 1970). It binds Ca^{2+} at high and low affinity sites and has been implicated in Ca^{2+} transport across the sarcoplasmic reticulum membranes by virtue of which it is able to play a major role in sequestering cytoplasmic Ca^{2+} ions in contracting muscle (Martonosi, 1971; MacLennan,

1974; Katz *et al.*, 1977). Some of the properties of this enzyme are summarised in Table 1.8.

1.5.3. Plasma membrane Na^+, K^+-ATPase

1.5.3.1 Components of the enzyme

The Na^+, K^+-ATPase of the plasma membrane plays an important role in the coupled transport of Na^+ and K^+ across animal cell membranes (Skou, 1965; Glynn, 1968). Although considerable information on the role of the Na^+, K^+-ATPase has accumulated since its discovery by Skou (1957), it has not been completely purified, although partial purifications have been claimed (Jorgensen *et al.*, 1971; Shamoo *et al.*, 1974; Shamoo and Goldstein, 1977).

The most purified enzyme is resolved into two polypeptides: the larger polypeptide has a molecular weight in the range of 85,000-135,000 daltons (Hokin *et al.*, 1973; Shamoo and Myers, 1974); whilst that of the smaller polypeptide is known to be between 40,000 and 50,000 daltons (Shamoo and Myers, 1974; Shamoo *et al.*, 1974).

1.5.3.2 Properties of the enzyme

The activity of the enzyme is stimulated maximally by Na^+ and K^+ in the presence of Mg^{2+}, as Mg^{2+}-ATP is its effective substrate (Skou, 1965); it is inhibited specifically by ouabain (Glynn, 1968). The enzyme has two cation binding sites: one site has a high affinity for Na^+ (4 to 6 times that of K^+) and the other a high affinity for K^+ (60 to 100 times that of Na^+) (Skou, 1960; Post *et al.*, 1965; Skou and Hillberg, 1969; Shamoo and Brodsky, 1972; see also Shamoo and Goldstein, 1977). When the enzyme is membrane-bound, it is activated by Na^+ from inside and by K^+ from outside (Garrahan and Glynn, 1967). The number of cations bound to each site has not been estimated. However, it is known that for the hydrolysis of one mole of ATP, 3 moles of Na^+ are transported in the

Table 1.9

Comparison of subunit composition of ATPases from different sources

ATPase PREPARATION		REFERENCES	NUMBER OF SUBUNITS	MOLECULAR WEIGHTS	IONOPHORE ACTIVITY
Mitochondrial	F_1	Kagawa *et al.* (1976)	5	56,000; 53,000; 32,000; 15,500; 11,000	F_o (H^+ conductor)
	F_o	Sone *et al.* (1975)	3	19,000; 13,500; 5,400	
Sarcoplasmic reticulum		Shamoo *et al.* (1976)	1	100,000	20,000 tryptic fragment (Ca^{2+}-ionophore)
Na^+, K^+-ATPase		Shamoo *et al.* (1974) Shamoo & Goldstein (1977)	2	94,000; 45,000	45,000 dalton (Na^+-ionophore)

outward direction and 2 moles of K^+ transported inwardly (Glynn, 1968; Hilden *et al.*, 1974), although these ratios vary in plasma membranes from different cellular sources.

The Na^+-dependent ionophoretic activity has been localised in a 45,000 dalton tryptic fragment of the enzyme and the ATP-hydrolysing activity in a 85,000 dalton fragment (Shamoo and Ryan, 1975). Since these two subunits are close to each other in the enzyme complex, it has been suggested that the enzyme utilizes energy provided by the hydrolysis of ATP to support its transport function (e.g., Na^+ transport) (Shamoo and Myers, 1974; Shamoo and Goldstein, 1977).

1.5.4 Conclusion

Three different ATPases (from mitochondria, sarcoplasmic reticulum and plasma membrane) which share some properties at least in common, have been described in this section. If a careful analysis of the individual molecular components of these enzymes is made, as shown in Table 1.9, it is apparent that they all possess a subunit that is involved in transport across the membrane in which they are present (e.g., transport of H^+, Ca^{2+} or Na^+). Furthermore, the subunit which has the ionophoretic activity is always found in close association with the subunit that has ATP-hydrolysing activity. This can be seen clearly in the case of the sarcoplasmic reticulum enzyme, which suggests that the energy of hydrolysis of ATP is utilised economically by the ionophore-like subunit for driving ions across the membranes. Since a similar kind of ATPase is described in this thesis, an ion-translocating function can also be ascribed to it, and this property will be discussed in Chapter 5.

1.6 CONTROL OF CELL Ca^{2+} AND THE POSSIBLE INVOLVEMENT OF ATPases

1.6.1 Introduction

The first part of the experimental work described in this thesis deals with the stimulation of pyruvate oxidation by Ca^{2+} in rat lymphoid tissue mitochondria. Recent work in several laboratories has shown that cytoplasmic Ca^{2+} is elevated passively and transiently during the transformation of lymphocytes treated with the plant lectin concanavalin-A or the Ca^{2+}-ionophore, A23187 (Freedman *et al.*, 1975; Hume and Weidemann, 1978). To determine whether an increase in cytoplasmic Ca^{2+} of this magnitude is sufficient to stimulate mitochondrial functions (e.g., pyruvate oxidation), the control of cellular Ca^{2+} movements by various mechanisms is considered briefly in this section. A major emphasis is placed on the ATPases of the sub-cellular systems and their possible involvement in the regulation of the Ca^{2+} level in the cytoplasm of mammalian cells.

1.6.2 Regulation of cell Ca^{2+} at the plasma membrane level

While it was first believed that the cytoplasmic Ca^{2+} concentration is regulated mainly by movements between intracellular Ca^{2+}-pools, expecially as a result of sequestration by mitochondria (Bygrave, 1967) and by the sarcoplasmic reticulum (Hasselbach, 1964; Ohnishi and Ebashi, 1963), more recent observations have favoured additional control at the level of the plasma membrane (Dietze and Heppe, 1971; Sacktor, 1977). Baker *et al.* (1969), for instance, observed a coupling between Ca^{2+} influx and Na^{+} efflux from the axons of *Loligo forbesi*. The enzyme that catalyzed this exchange reaction was found to be insensitive to ouabain and therefore clearly distinguishable from the ouabain-sensitive Na^{+}, K^{+}-pump. Increased internal Na^{+} or decreased external Na^{+} promoted the entry of

Ca^{2+} into cells in exchange for internal Na^{+} in the ratio of 1:4, while Ca^{2+} efflux was enhanced by elevated external Na^{+} or by decreased external Ca^{2+} concentrations. It was suggested on the basis of these observations that, in an exchange between internal Ca^{2+} and external Na^{+}, some of the energy for extruding Ca^{2+} is provided by the downhill movement of Na^{+}. These findings are consistent with the view that, apart from exchange-diffusion of Ca^{2+} across the plasma membrane, there is a more direct coupling between Ca^{2+} efflux and some component of Na^{+} influx (Blaustein and Hodgkin, 1969). This mechanism of Ca^{2+} extrusion is different from the energy-dependent extrusion of Ca^{2+} from red blood cells that is driven by a Ca^{2+}-activated ATPase (Schartzmann, 1967). These findings suggest that a Ca^{2+} - Na^{+} exchange mechanism could participate in maintaining the internal concentration of Ca^{2+} at a sufficiently low level to maintain normal function without the direct intervention of metabolism or consumption of ATP (Baker *et al.*, 1969; Blaustein and Hodgkin, 1969).

A Ca^{2+}-stimulated ATPase which is involved in the active transport of Ca^{2+} has been identified in the sarcolemmal membrane of myocardium (Sulakhe and Dhalla, 1971; Dietze and Heppe, 1971), renal plasma membranes (Kinne-Saffran and Kinne, 1974) and intestinal brush borders (Moore *et al.*, 1974). In the sarcolemmal membranes of myocardium it has been shown that the activity of the Ca^{2+}-ATPase and Ca^{2+} influx are stimulated simultaneously by c-AMP (Dietze and Heppe, 1972). Janke *et al.* (1970) have shown in the same system that hormones such as adrenalin, which enhance c-AMP production, also induce Ca^{2+} influx. Together, these observations indicate that the Ca^{2+}-stimulated ATPase of sarcolemmal membrane may catalyse active, hormone-sensitive Ca^{2+} transport across the plasma membrane. The possible involvement of this enzyme in the regulation of cytoplasmic Ca^{2+} levels in tissues such as heart and smooth muscle, which lack an extensively developed sarcoplasmic reticulum, is an attractive hypothesis.

Table 1.10

Proteins of sarcoplasmic reticulum membranes

PROTEIN	PROPERTIES	REFERENCES
Ca^{2+}-ATPase (major protein)	Refer to the text	Stewart and MacLennan, (1974)
Low molecular weight proteolipid	Associated closely with ATPase; water-insoluble; properties not known	MacLennan *et al.* (1972)
Calsequestrin	Highly acidic protein; binds large quantities of Ca^{2+} (35 to 43 moles/ mole).	MacLennan & Wong (1971); Oswald & MacLennan (1974); MacLennan (1974)
Mol. wt 55,000 dalton protein	High-afinity Ca^{2+}-binding protein different from tryptic digestion product of ATPase	MacLennan *et al.* (1972)

1.6.3 Regulation of cell Ca^{2+} by the sarcoplasmic reticulum

The sarcoplasmic reticulum membranes are specialised for the transport and binding of Ca^{2+} in skeletal muscle and it is possible that the endoplasmic reticulum may play a similar role in non-muscle cells (Weber, 1966; Ebashi *et al.*, 1969; Martonosi, 1971; MacLennan, 1970; Katz *et al.*, 1977a). As the sarcoplasmic reticulum membrane is highly specialised for Ca^{2+} transport, it contains relatively fewer proteins (MacLennan, 1970; Martonosi and Halprin, 1971) and the major ones are listed in Table 1.10. In addition, a group of acidic proteins, which bind a large amount of Ca^{2+} with low affinity (Stewart and MacLennan, 1974), have been identified in sarcoplasmic reticulum membranes (Oswald and MacLennan, 1974), although their function has not been clearly defined.

Sarcoplasmic reticulum vesicles have been shown to transport Ca^{2+} actively in the presence of Mg^{2+} and ATP (Hasselbach and Makinose, 1961). When the ionised Ca^{2+} concentration is in the micro-molar range in the external medium, the vesicles rapidly accumulate 100 nmols Ca^{2+}/mg of protein (Ebashi and Lipman 1962; Weber *et al.*, 1966; Katz *et al.*, 1977b). In the presence of Ca^{2+}-precipitating ions, such as oxalate and P_i, the vesicles take up and sequester an even larger amount of Ca^{2+} (Hasselbach and Makinose, 1963; Ogawa, 1970). These anions increase net Ca^{2+} transport by maintaining a low free Ca^{2+} concentration inside the vesicles, thereby reducing the inhibitory effect of high internal Ca^{2+} on the uptake process (Weber, 1971).

The direct involvement of the (Ca^{2+} + Mg^{2+}) -ATPase of sarcoplasmic reticulum in Ca^{2+}-transport has been convincingly demonstrated in experiments where the purified enzyme has been incorporated into phospholipid vesicles and an ATP-dependent Ca^{2+}-uptake demonstrated (Racker, 1972; Racker *et al.*, 1975). The same results indicate that the Ca^{2+}-transporting ionophoretic site resides within the enzyme molecule (see chapter 1.5.2.1 and Table 1.9), which gives further support to the proposal that the ATPase

is the "primary machine" for active Ca^{2+}-transport by sarcoplasmic reticulum.

Various factors which alter the activity of the sarcoplasmic reticulum ATPase have been shown to influence also the Ca^{2+} movements into the organelle. Mercurials which inhibit the enzyme (Shamoo and Goldstein, 1977) and bivalent cations such as Zn^{2+} and Mn^{2+}, which compete with Ca^{2+} for binding sites on the purified enzyme, also inhibit Ca^{2+} uptake (Shamoo and Goldstein, 1977). It has been shown that c-AMP, which increases oxalate-dependent Ca^{2+} uptake into some cardiac sarcoplasmic reticulum preparations (Entman *et al.*, 1969; La Raia and Morkin, 1974), appears to exert its effect by stimulating the Ca^{2+}-activated ATPase as a result of protein kinase-dependent membrane phosphorylation (Wray *et al.*, 1973; Wray and Gray, 1977).

Sarcoplasmic reticulum has been shown to have only one major enzymic activity, a Ca^{2+}-dependent ATPase; and only one function, Ca^{2+} binding and release (Ostwald and MacLennan 1974; Katz *et al.*, 1977a). It controls the concentration of cytoplasmic Ca^{2+}, thereby controlling contraction and relaxation of muscle (Weber, 1966), and any other cytosolic function that depends critically on Ca^{2+}.

1.6.4 Regulation of cell Ca^{2+} by mitochondria

The energy-linked uptake of Ca^{2+} by mitochondria, first described in the early 1960's (see Deluca and Engstrom, 1961; Vasington and Murphy, 1962; Saris, 1963; Brierley *et al.*, 1964), can be supported by energy derived from mitochondrial respiration (Chance, 1956; Rossi and Lehninger, 1964), by valinomycin-induced K^+ efflux in respiration-inhibited mitochondria (Scarpa and Azzone, 1970) or by the hydrolysis of ATP (Bielawski and Lehninger, 1966; Spencer and Bygrave, 1973). Although these forms of energy are diverse (e.g., redox, phosphate group transfer and electrochemical - see Bygrave, 1977), the experiments have established clearly

Table 1.11

Properties of Ca^{2+}-transport system in mitochondria

(from Bygrave, 1977)

Location	inner membrane
Concentration	Only several p mols/mg protein
Velocity (at 0°C) of Ca^{2+} uptake	About 0.5 ± 0.1 nmoles/sec/mg protein or 1.0 ± 0.01 nmoles/sec/mg protein in the presence of 2mM P_i
Affinity for free Ca^{2+}	2-4μM
Competitive inhibitor	La^{3+} ($K_i \simeq 2 \times 10^{-8}$ M)
Non-competitive inhibitor	Ruthenium Red ($K_i \simeq 3 \times 10^{-8}$ M)
Metal ion specificity	$Ca^{2+}>Sr^{2+}>Mn^{2}>Ba^{2+}>Fe^{2+}>La^{3+}$
pK_a of binding groups	7.5
Minimal energy requirement	Membrane potential

that a electrochemical proton gradient across the inner mitochondrial membrane is the principal driving force for the translocation of Ca^{2+} (see Bygrave, 1977). Moreover, anions that are translocated by mitochondria also distribute themselves across the inner membrane in accordance with this proton gradient (Quagliarello and Palmieri, 1970; McGivan and Klingenberg, 1971).

With the discovery of high-affinity Ca^{2+} binding sites (Reynafarje and Lehninger, 1969) that can be competitively blocked by La^{3+} (Mela, 1968), the existence of a Ca^{2+}-binding protein has been identified and isolated from a variety of mitochondria (Lehninger, 1971; Sottocasa *et al.*, 1972; Carafoli and Sottocasa, 1974; Sandri *et al.*, 1976). Some of the properties of the Ca^{2+}-transport system in mitochondria are summarised in Table 1.11. The principal factor that regulates the influx and efflux of Ca^{2+} from mitochondria is the concentration of the Ca^{2+}-ion itself (Reed and Bygrave, 1975; Bygrave, 1975; Valliers *et al.*, 1975). Several other factors, such as c-AMP (Borle, 1974), phosphoenol-pyruvate (Peng *et al.*, 1974), P_i (Harris, 1972), adenine nucleotides (LeBlanc and Clauser, 1974) and Na^+ (Carafoli *et al.*, 1974) have been shown to influence the movement of Ca^{2+} ions in an indirect fashion (see Bygrave, 1977). Certain cations (e.g., Mg^{2+}), P_i and the intramitochondrial concentration of adenine nucleotides act as stabilizing components for the Ca^{2+} that has been taken up by mitochondria (Siliprandi *et al.*, 1975; see Bygrave, 1977).

By virtue of their Ca^{2+} transporting and sequestering ability, mitochondria are able to play a major role in the modification of cell metabolism by controlling the intracellular ionic environment (see Gevers and Krebs, 1966; Bygrave, 1977), since a large number of enzymes and metabolic pathways are sensitive to fluctuations in the concentration of cytoplasmic Ca^{2+} (Bygrave, 1967, 1976; Rasmussen, 1970). A detailed discussion of other possible physiological roles for Ca^{2+} movements in

different tissues is beyond the scope of this chapter.

1.7 CONTROL OF CELL Ca^{2+} IN RELATION TO CELL ACTIVATION

A large number and variety of enzymes are sensitive to low concentrations of Ca^{2+}: this is seen most clearly with cytoplasmic enzymes such as pyruvate kinase (Bygrave, 1966; Meli and Bygrave, 1972) and mitochondrial enzymes such as pyruvate dehydrogenase (Linn *et al.*, 1969a, b; Randle *et al.*, 1974; Denton *et al.*, 1975) and pyruvate carboxylase (Foldes and Barritt, 1977). Many metabolic pathways, such as protein biosynthesis (Rao *et al.*, 1974) and pathways of phospholipid metabolism (Roberts and Bygrave, 1973), have been shown to be influenced by Ca^{2+}. Moreover, events like the antigen-triggered release of histamine from mast cells (Mongar and Schield, 1958), the release of catecholamines from the chrommafin cells of adrenal medulla (Douglas and Rubin, 1961) and the secretion of hydrolases by neutrophils (Woodin and Wieneke, 1963) have been shown to be absolutely dependent on extracellular Ca^{2+}.

From the point of view of this thesis, which is concerned with lymphoid tissue metabolism, the exact regulatory role of Ca^{2+}-ions in the initiation of the transformation of T-lymphocytes (Whitney and Sutherland, 1972, 1973; Parker, 1974) is considered in greater detail. Freedman *et al.* (1975) have described a "gated Ca^{2+}-flux" across the plasma membrane into T-lymphocytes in response to the addition of mitogenic plant lectins (e.g., concanavalin-A) at optimum concentration. They proposed that a large influx of extracellular Ca^{2+} into the cells is completed within one minute. In contrast, a large and continuous increase in Ca^{2+} uptake has been reported by Whitney and Sutherland (1973). Similarly,

increased Ca^{2+} uptake by lymphocytes has been observed or implicated in cell activation during ionophore A23187 or phytohaemagglutinin (PHA) treatment (Raff *et al.*, 1975; Greene *et al.*, 1976; Culvenor and Weidemann, 1976).

However, recent findings by Metcalf and his co-workers have raised the possibility that "lymphocytes cannot become committed to transform while the Ca^{2+} influx is maintained" (Hesketh *et al.*, 1977). They suggest that the observed increase in Ca^{2+} influx due to concanavalin-A, ionophore A23187 or PHA treatment, which is reported to result in cytoplasmic concentrations of 0.5 to 300 mM (Parker, 1974; Freedman *et al.*, 1975), could be cytotoxic, since Ca^{2+} present in the cytoplasm at even 1.0 mM would inhibit glycolysis and protein synthesis. These two pathways are both considerably stimulated in transformed cells (Roos and Loos, 1973; Culvenor and Weidemann, 1976). Although Metcalf has questioned the requirement for a shortlived uptake of extracellular Ca^{2+} during the early stages of lymphocyte stimulation, his results do not exclude the possibility that "a small increase in cytoplasmic Ca^{2+} is a component of the signal that commits the cell to transform" (see Hesketh *et al.*, 1977).

If Ca^{2+} uptake does play an essential role in lymphocyte transformation, it is of importance to establish whether there is a direct causal relationship between the observed Ca^{2+} fluxes and the metabolic changes that are associated with DNA synthesis during lymphocyte transformation (Wang *et al.*, 1976). It has been demonstrated, in both spleen and thymus, that the glucose carrier, hexokinase, phosphofructokinase and pyruvate kinase, but no other glycolytic reactions, are displaced significantly from thermodynamic equilibrium *in vivo* (see Suter, 1973; Culvenor and Weidemann, 1976). The acceleration of 3-o-methyl-D-[U-^{14}C]-glucose transport in the presence of concanavalin-A (but not the unstimulated basal transport rate) is dependent in a sigmoidal manner on the free Ca^{2+} concentration in the external medium (Yasmeen *et al.*, 1977). This is also

true in the absence of concanavalin-A if the Ca^{2+} ionophore A23187 is used as the stimulating agent (Hume and Weidemann, 1978). Hence, an increased flux of carbon through the glycolytic pathway could be attributed solely to the activation of the glucose carrier (Culvenor and Weidemann, 1976; Yasmeen *et al.*, 1977). A crucial unsolved problem is whether the Ca^{2+} uptake that preceeds detectable changes in the rate of glucose transport is responsible for setting these changes in motion or whether it is an independent, but simultaneous, consequence of mitogen-binding.

As a result of the stimulation of glucose transport by either concanavalin-A or A23187, a 4-to-5-fold increase in lactate plus pyruvate production has been detected in lymphocytes within 30 minutes; in the same experiment the oxidation of [U-^{14}C]-glucose to $^{14}CO_2$ increased by only 30-50% (Yasmeen *et al.*, 1977). Earlier studies on spleen slices (Suter and Weidemann, 1975, 1976) and isolated thymus cells (see Chan, 1972) have indicated that the oxidation of pyruvate by lymphoid tissue mitochondria is limited by the very low activity of pyruvate dehydrogenase, which is a non-equilibrium enzyme (Suter, 1973). Its activity is considerably slower than the rate of pyruvate production by the glycolytic pathway and its K_m is below the prevailing steady-state concentration of pyruvate in cells metabolising 5mM glucose. It is likely that an increase in pyruvate oxidation by this tissue would require the activation of pyruvate dehydrogenase by factors other than an increase in the steady-state concentration of its substrate.

It is worth considering in this context that the activity of pyruvate dehydrogenase in adipose tissue is regulated by a phosphorylation-dephosphorylation mechanism (Denton *et al.*, 1975). Randle and his co-workers and Wieland have suggested that Ca^{2+} ions may play a crucial role in the activation of pyruvate dehydrogenase, by virtue of the ability of Ca^{2+} to stimulate PDH-phosphate phosphatase (Wieland *et al.*, 1972; Severson *et al.*, 1974). The present work was undertaken to ascertain whether a

rise in cytoplasmic Ca^{2+}, to the extent that occurs during lymphocyte stimulation, is a sufficient stimulus to explain the activation of pyruvate oxidation observed in whole cells.

Mitochondria from a number of tissues including liver, kidney and heart (Rasmussen, 1970; Borle, 1974; Carafoli, 1974; Bygrave, 1977; see also Chapter 1.6.4) are able to contribute to alterations in the intracellular distribution of Ca^{2+}. In contrast, very little is known about mitochondria from lymphoid tissues and their possible role in the cellular redistribution of Ca^{2+}. Mitochondria were first isolated from spleen by Potter and Bethel (1952) and their oxidative phosphorylative activities were tested after X-irradiation (Maxwell and Ashwell, 1953; Eichel, 1957). Similar studies were made with thymus mitochondria (Scaife, 1966) which were isolated in sucrose medium (0.25M) supplemented with Ca^{2+} (3.0mM). Most of the available reports on these mitochondria describe the deleterious effects of X-irradiation on their oxidative functions (Ashwell and Hickman, 1952; Cole *et al.*, 1952; Maxwell and Ashwell, 1953; van Bekkum, 1955; Mantifel and Meisel, 1965; Scaife, 1966). No other systematic study of the properties of mitochondria isolated from lymphoid tissues have been carried out.

In our hands, when spleen mitochondria were isolated in an iso-osmotic sucrose medium, addition of ADP (in an incubation medium containing substrates and Mg^{2+}) stimulated state-3 respiration in such a manner that it did not cease when the added ADP was exhausted. When spleen mitochondria were isolated in the same medium supplemented with EGTA and were then incubated in a medium free of added bivalent metal ions (e.g., Mg^{2+}), they were tightly-coupled and gave high respiratory control and ADP/O ratios. These observations can be explained if there is a bivalent metal-ion-stimulated ATP-hydrolysing reaction in rat spleen mitochondria that is accessible to exogenous Mg^{2+}-ions and continuously regenerates ADP. The

results of detailed investigations of the properties of this reaction are presented in the following chapters.

1.8 AIMS OF THIS PROJECT

In view of the importance of bivalent metal ions, especially Ca^{2+}, in lymphocyte stimulation this project was planned with the following objectives:

(1) to elucidate whether a rise in the steady-state cytoplasmic Ca^{2+} concentration, which occurs transiently during lymphocyte stimulation, has the capacity to increase mitochondrial pyruvate oxidation to the extent observed in stimulated lymphocytes;

(2) to determine systematically the nature and location of the ATP hydrolysing system (ATPase) present in the EGTA-accessible space of spleen mitochondria; and

(3) to investigate the kinetic properties of this enzyme with a view to determining its possible role in the regulation of the energy-linked functions of spleen mitochondria and the Ca^{2+} homeostasis of the cells that contain it.

In the present study, attention has been focussed on the ATP-hydrolysing system (ATPase) from the outer membrane fraction of rat spleen mitochondria. Although a similar enzyme has been reported in mitochondria from tissues like heart and kidney-cortex, no systematic study of its functional role in those tissues has been undertaken. In this project, I have limited myself to describing a functional role for the outer membrane ATPase, and possibly a role for the "outer mitochondrial compartment", in general terms - with particular emphasis on the regulation of Ca^{2+}-ion movements in the lymphocyte subpopulations of the spleen that seem to contain it.

CHAPTER 2

REGULATION OF PYRUVATE OXIDATION BY BIVALENT CATIONS IN RAT LYMPHOID TISSUE MITOCHONDRIA

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2.1 INTRODUCTION

Concanavalin-A is a mitogenic protein isolated from the Jack bean *Canavalia ensiformis* which has the ability to transform "resting" small lymphocytes, in a non-specific fashion, into lymphoblasts capable of mitosis. The Ca^{2+}-ionophore A23187, which is a product of the genus *Streptomyces*, is a mobile carrier of Ca^{2+}-ions (Pressman, 1968; Gomperts, 1976) that is capable of mimicking, to some extent, the action of concanavalin-A in stimulating lymphocytes.

Prominent among the early metabolic events that occur during thymus lymphocyte transformation is an increased uptake of Ca^{2+}-ions and [$U^{14}C$]-glucose from the incubation medium and a subsequent stimulation of glycolysis and $^{14}CO_2$ production (see Chapter I). This chapter is devoted primarily to examining whether the increased production of CO_2 from glucose oxidation in transforming lymphocytes is a direct consequence of increased mitochondrial pyruvate oxidation. Secondly, in accordance with the principles discussed in Chapter I, it is aimed at determining whether Ca^{2+}-ions, which are thought to play a "triggering" role in lymphocyte stimulation (Gomperts, 1976), are capable of stimulating pyruvate oxidation in isolated mitochondria, as Ca^{2+}-ions have been implicated in the activation of pyruvate dehydrogenase in a number of tissues (Linn *et al.*, 1969a, b; Severson *et al.*, 1974; Denton *et al.*, 1975).

As a first approach to testing this hypothesis, intact mitochondria were isolated from lymphoid tissues (rat spleen and thymus) and their ability to oxidize pyruvate and other oxidizable substrates was tested in the presence and absence of added bivalent metal ions.

2.2 MATERIALS

[U-^{14}C]-glucose, [6-^{14}C]-glucose and [1-^{14}C]-pyruvate were obtained from the Radiochemical Centre, Amersham, U.K. [1-^{14}C]-pyruvate was dissolved in water, stored in aliquots at -20°C and used within 14 days of receipt. All enzymes used in substrate assays were from Boehringer, Mannheim, GmbH, West Germany. $CaCl_2$ was a 100mM standard solution from Orion Research Inc., MA., U.S.A. Ionophore A23187 was from Lilly Research Laboratories, Indianapolis, IN., U.S.A. and was dissolved in ethanol before use. Butyl-PBD[5-(4-biphenylyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole], was from Koch-Light Laboratories, Colinbrook, U.K. and hyamine hydroxide (a 1.0 M solution in methanol) was from Packard Instrument Co., Dowers Grove, Ill., U.S.A. Concanavalin-A was prepared from Jack bean meal by the method of Agarwal and Goldstein (1967) and was stored as an aqueous suspension at -20°C. All other reagents used were of the highest purity available commercially. Rubber "Suba-seals" (size 33, to fit Beckman glass scintillation vials) were from Townson and Mercer, Lane Cove, N.S.W., Australia.

2.3 METHODS

2.3.1 Preparation of thymus lymphocytes

Pooled thymus cells from seven week-old outbred male Wistar rats were prepared at room temperature by teasing thymus tissue with 22-gauge needles in phosphate-buffered saline (NaCl, 136mM; KCl, 4.7mM; $CaCl_2$, 1.3mM; $MgSO_4$, 0.1mM; KH_2PO_4, 1.2mM; Na_2HPO_4, 4.0mM; NaH_2PO_4, 1.0mM) at pH 7.2 (Krebs and de Gasquet, 1964). Cells were gently dispersed with a pasteur pipette, centrifuged for 30 seconds at 50×g to remove debris and then washed by centrifuging at 300×g for 7 minutes. They were finally resuspended in a suitable volume of the same buffer. The viability of the cells, determined as described by Culvenor and Weidemann (1976), was over 95%.

2.3.2 Incubation procedure

Incubations were carried out at 37°C. Cells were suspended at a density of 1.3×10^8/ml in a final volume of 3.0ml in Warburg flasks containing the P_i-buffered saline (pH 7.2) described above, plus the additions indicated in the Legends. The cells were gassed with oxygen (100%) for 30 seconds and the reactions initiated by addition of radioactive glucose alone (5.0mM) or by simultaneous addition of glucose and either mitogen or A23187. Reactions were terminated by addition of HCl (0.5ml; 0.5M) and the $^{14}CO_2$ liberated was collected in removable centre-wells containing CO_2-free KOH (2M; 200µl) by shaking the flasks for a further 10 minutes. They were then cooled to 0°C rapidly and treated with perchloric acid (0.5ml; 3.0M) to precipitate the protein. The centre-wells were removed, rinsed clean on the outside and placed into sealed tubes containing CO_2-free water (4.8ml). $^{14}CO_2$ was estimated by pipetting a small sample of this solution (200µl) into 10ml of scintillation fluid (Butyl PBD, 15g; toluene, 1.5 litres and methoxy ethanol, 1.0 litre) and counting in a Beckman LS-350 liquid scintillation counter. Total CO_2 was assayed in 2.0 ml samples of the diluted centre-well contents by the manometric method of Weidemann and Krebs (1969). The protein precipitated in the remaining cell suspension was removed by centrifugation (27,000 × g for 5 minutes) and the supernatants were neutralised with K_2CO_3 (5M) and made up to 5.0ml. Aliquots of this solution were then assayed for substrates and products.

2.3.3 Analytical methods

Pyruvate was assayed by a fluorometric adaptation of the method of Bücher *et al.* (1965); lactate by the method of Hohorst (1965); glucose by the method of Slein (1965); acetoacetate and 3-hydroxybutyrate by the method of Williamson *et al.* (1962); aspartate by the method of Pfeiderer (1965); glutamate by the method of Bergmeyer and Bernt (1965) and glycogen by the method of Bartley and Dean (1968).

[^{14}C]- glucose, [^{14}C]-glutamate, [^{14}C]-aspartate and ^{14}C-lactate derived from [U-^{14}C]-glucose were separated and analysed as described by Hume *et al.* (1978). Extraction of DNA, RNA and lipids from cell suspensions was carried out as described by Hume *et al.* (1978).

2.3.4 Measurement of ^{45}Ca uptake by rat thymus lymphocytes

Suspensions of rat thymus lymphocytes were prepared as described in 2.3.1. The initial rate of ^{45}Ca uptake was measured in thymocytes prepared in the phosphate-buffered saline of Krebs and de Gasquet (1964) modified to contain no Ca^{2+}. The cells (3.3×10^8/ml) were incubated at 37°C in glass vials containing glucose and acetoacetate (both at 5mM), phosphate-buffered saline (with the Ca^{2+}-concentration reduced to 0.1mM) and appropriate additions in a total volume of 1.2ml. The low Ca^{2+} concentration used was close to saturation for the uptake process (L.M. Russell, D.A. Hume and M.J. Weidemann, unpublished results) and was chosen to avoid excessive dilution of the specific activity. After at least 15 minutes preincubation, reactions were started by simultaneous addition of ^{45}Ca (approx. 0.1µCi) and concanavalin-A, A23187 or saline. Samples (0.1ml) were removed at 1 minute intervals, layered on to a bovine serum albumin cushion (1.0ml, 10% bovine serum albumin fraction V in 0.9% NaCl, pH 7.4) and centrifuged immediately in an Eppendorf bench centrifuge for 30 seconds. The supernatants were aspirated off and the pellets were dissolved in HCOOH for radioactive counting. Initial rates derived from

the linear portion of the uptake curve. The rate of 3-o-methyl [U-^{14}C]-glucose uptake by rat thymus lymphocytes was estimated as described by Yasmeen *et al.* (1977).

2.3.5 Isolation of mitochondria

Male albino Wistar rats were stunned with a blow on the head and exsanguinated. The spleens or thymuses were removed quickly and placed in an ice-cold isolation medium containing sucrose (0.25M), EGTA (1mM), bovine serum albumin (1% w/v) and Hepes (5.0mM; pH 7.4). The pH of the medium was adjusted to pH 7.4 with KOH prior to use. The pooled spleens or thymuses (approx. 5g tissues) were minced coarsely with scissors and suspended in 9 volumes of isolation medium. The mince was homogenised with two passes of a motor-driven Potter-Elvehjem tissue homogenizer. The homogenate was centrifuged at 650 × g for 10 minutes and the pellet discarded. The supernatant was centrifuged at 8,500 × g for 15 minutes, and the resulting mitochondrial pellet was washed twice (with isolation medium free of EGTA) and centrifuged between washes at 8,500 × g to resediment the pellet. The mitochondrial pellet was resuspended finally in a suitable volume of the same wash medium to give a final protein concentration of 20mg/ml.

2.3.6 Measurement of mitochondrial respiration

Respiration was measured with a Clarke-type oxygen electrode (Reed, 1972). The reaction mixture in a final volume of 2.0ml consisted of sucrose (0.25M), EDTA or EGTA (1.0mM), potassium phosphate buffer (10mM; pH 7.4), bovine serum albumin (1% w/v), Hepes (10mM; pH 7.4), 0.5-0.8mg of mitochondrial protein and, where applicable, $MgCl_2$ or $CaCl_2$ at the concentrations indicated in the legends to the Tables and Figs. The terminology of Chance and Williams (1956) for various mitochondrial states and for respiratory control ratios is used throughout.

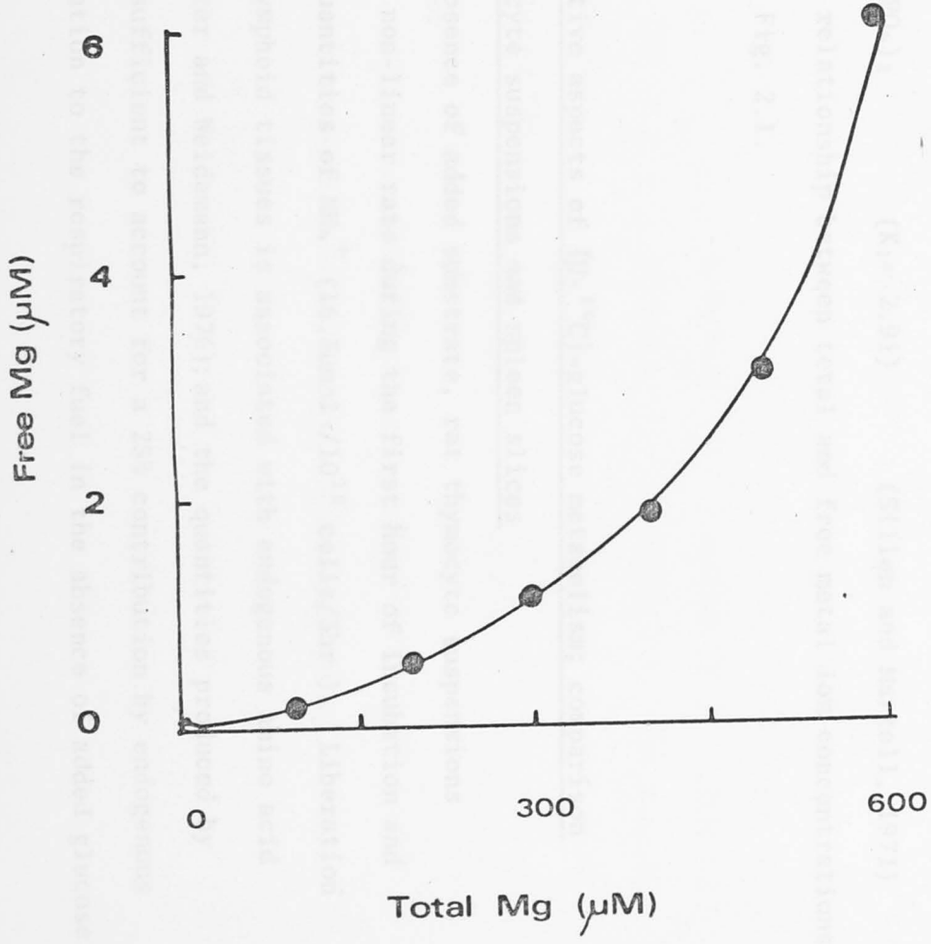
2.3.7 Measurement of protein

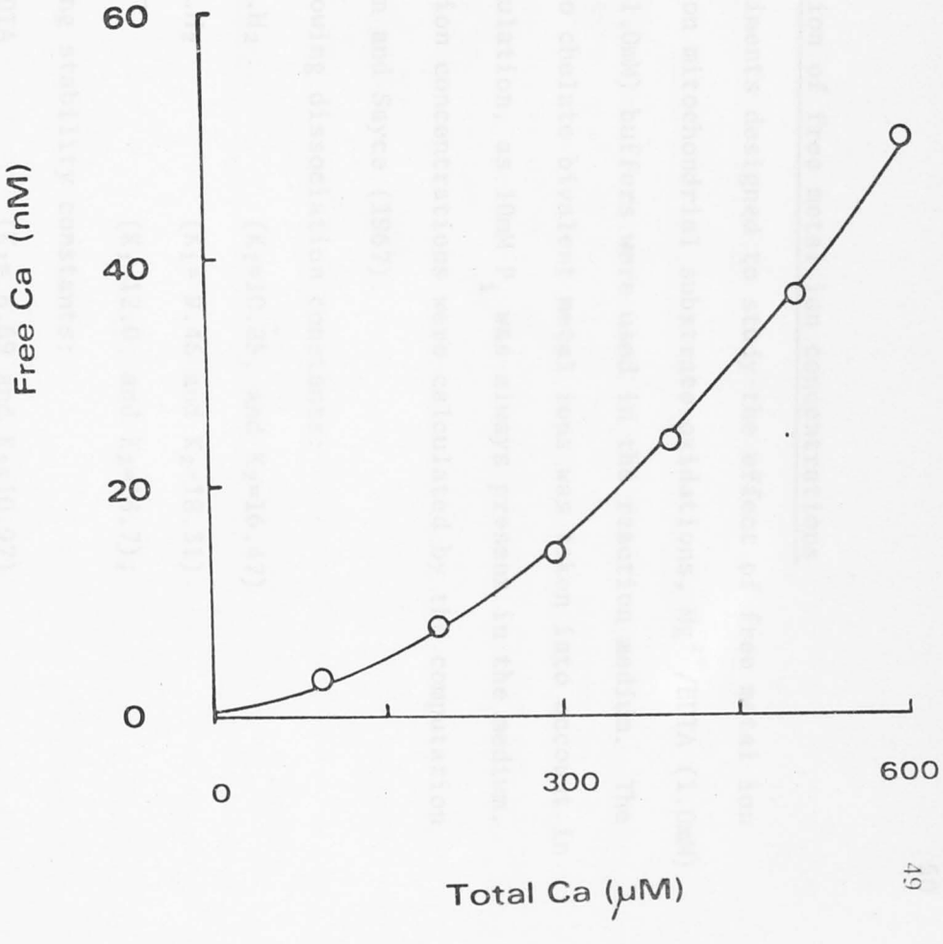
Mitochondrial protein was determined by the Biuret method of Layne (1957), with bovine serum albumin as the protein standard.

2.3.8 Mitochondrial [1-^{14}C]-pyruvate oxidation

The oxidation of [1-^{14}C]-pyruvate by mitochondria isolated from rat spleen and thymus was studied in a reaction medium similar to that used for measuring mitochondrial respiration but containing malate (0.1mM) and ADP (5.0mM) in addition in a total final volume of 1.0ml. Mitochondria (1.4mg protein in the case of spleen and 0.5mg protein in the case of thymus) were added to a reaction medium, in Beckman glass scintillation vials, containing the desired concentrations of Ca^{2+} (as indicated in the Legends), and the mixture was preincubated at room temperature for 5 minutes before being placed on ice. The vials were sealed tightly with "Suba seals", after placing an open Eppendorf tube inside, and incubated at 37°C in a shaking water bath. The reaction was initiated by injecting sufficient [1-^{14}C]-pyruvate (specific activity; 0.1μCi/μmol) through the seal to give a final concentration of 1.0mM in the reaction medium. At the end of 5 minutes, the reaction was stopped with an injection of citric acid (200μl, 65% w/v). The $^{14}CO_2$ liberated inside the vials was collected by injecting hyamine hydroxide (1.0ml) into the Eppendorf tubes and shaking the vials gently for a further 30 minutes. To ensure maximum $^{14}CO_2$ trapping in the hyamine hydroxide, saturated $NaHCO_3$ (200μl) was injected into the acidic incubation mixture and the vials were shaken for a further 30 minutes, which improved both the recovery and reproducibility of the $^{14}CO_2$ assay. The Eppendorf tubes were then removed, rinsed with water, wiped clean and transferred to scintillation vials containing 10ml of scintillation fluid; the radioactivity was determined as described above. Control runs, in which mitochondria were replaced by an equal volume of water, were performed under identical conditions.

Fig.2.1 Calculation of free metal ion concentrations


Free Mg (μM)
6
4
2
0
0
300
600
Total Mg (μM)


Free Ca (nM)
60
40
20
0
0
300
600
Total Ca (μM)

2.3.9 Calculation of free metal ion concentrations

In experiments designed to study the effect of free metal ion concentrations on mitochondrial substrate oxidations, Mg^{2+}/EDTA (1.0mM) and Ca^{2+}/EGTA (1.0mM) buffers were used in the reaction medium. The ability of P_i to chelate bivalent metal ions was taken into account in making the calculation, as 10mM P_i was always present in the medium. The free metal ion concentrations were calculated by the computation method of Perrin and Sayce (1967).

The following dissociation constants:

for EDTA.H_2 (K_1=10.25, and K_2=16.47)

EGTA.H_2 (K_1= 9.46 and K_2=18.31)

and PO_4^{3-} (K_1=12.0 and K_2=18.7);

and the following stability constants:

for Mg/EDTA (K_1= 8.69 and K_2=10.97)

Ca/EGTA (K_1=10.97 and K_2=14.76)

$Ca_3(PO_4)_2$ (K_1= 6.3, K_2=14.77 and K_3=19.57)

and $Mg_3(PO_4)_2$ (K_1= 2.91) (Sillen and Martell, 1971)

were used. The relationship between total and free metal ion concentrations is presented in Fig. 2.1.

2.4 RESULTS

2.4.1 Quantitative aspects of [U-^{14}C]-glucose metabolism; comparison of thymocyte suspensions and spleen slices

In the absence of added substrate, rat thymocyte suspensions consume O_2 at a non-linear rate during the first hour of incubation and produce large quantities of NH_4^+ (16.3µmol /10^{10} cells/3hr). Liberation of ammonia by lymphoid tissues is associated with endogenous amino acid oxidation (Suter and Weidemann, 1976); and the quantities produced by thymocytes are sufficient to account for a 25% contribution by endogenous amino acid oxidation to the respiratory fuel in the absence of added glucose.

Table 2.1

The effects of concanavalin-A on the metabolism of [U-^{14}C]-glucose by rat thymocytes

The values reported here are from Hume *et al.* (1978). Rat thymus lymphocytes (1.3×10^8/ml) were incubated for three hours in the presence of [U-^{14}C]-glucose (5mM, 10^6cpm/flask)± A23187 (0.4µg/ml) or concanavalin A (50 µg/ml) in a total volume of 3ml. Details of substrate assays and calculations are presented in the text. Results for all substrates are presented as µmoles formed (or utilized)/10^{10} cells/3 hour incubation ± S.E.M. for the number of observations (in parentheses) on separate cell preparations.

	CONTROL	+CONCANAVALIN-A	+ A23187
	(7)	(7)	(5)
O_2 consumption	-412±20	-435±24	-424±34
CO_2 production	+416±27	+446±29	+436±36
Respiratory quotient (CO_2/O_2)	1.0±0.02	1.03±0.02	1.03±0.02
Glucose disappearance	-72.6±7.3	-141.3±6.9	-109.9±16.7
Lactate production	+47.8±3.5	+153.2±9.2	+117.9±19.7
Pyruvate production	+2.64±0.2	+ 5.43±0.32	+ 5.2±0.8
Cpm in $^{14}CO_2$ (x 10^{-4})	4.92±0.32	7.19±0.57	6.55±0.47
Specific activity of $^{14}CO_2$ (x 10^{-3})	3.36±0.23	4.60±0.37	7.21±0.22
Glucose to $^{14}CO_2$ (%)	27.7±3.1	20.7± 1.1	24.4 ±2.7
Glucose to lactate (%)	37.6±3.8	57.8±5.3	56.5 ±5.0
Glucose not accounted for (%)	33.6±5.4	21.5±4.9	19.9 ±6.2
Glucose to fuel of respiration (%)	43.0±3.2	57.7±4.6	50.8 ±3.9

The addition of glucose (5mM) leads to a 50% increase in O_2 consumption, which becomes linear, and an almost total suppression of NH_4^+ production (Beaver, 1975). In contrast, saturating concentrations of glucose do not stimulate O_2 consumption in rat spleen slices (Suter and Weidemann, 1975) and only partially suppress the observed NH_4^+ production. Isolated thymocytes may not have an endogenous fuel supply as large as that possessed by the metabolically active cell populations of spleen slices as they incur less mechanical damage during preparation, reducing the possibility of artefactual proteolysis.

The major quantitative difference between thymocytes and spleen slices in the fate of the glucose metabolised lies in the production of lactate and pyruvate, which accounts for 38% of the glucose disappearance in thymocytes (Table 2.1) but for 70-80% in spleen slices (Suter and Weidemann, 1975). The percentage of the glucose uptake accounted for by CO_2 production is approximately the same in the two preparations, leaving 34% of the glucose consumed by thymocytes unaccounted for by lactate, pyruvate and CO_2 production (Table 2.1). This figure is negligible in spleen slices (Suter and Weidemann, 1975). The most likely fate of the major part of the glucose not accounted for is glycogen. Puckle (1974) has shown that 24% of the c.p.m. recovered when thymocytes are incubated with [U-^{14}C]-glucose is found in glycogen and oligosaccharides, (see Table 2.2) which is a much larger percentage than that recovered in spleen slices. No net change in the tissue glycogen concentration is observed on incubation of spleen slices with glucose (Suter and Weidemann, 1975), but thymocytes apparently have a much greater capacity than spleen slices for glycogen synthesis.

Table 2.2

The effect of concanavalin-A on the fate of labelled carbon from [U-^{14}C]-glucose in rat thymocytes

The values are taken from Puckle (1974). The methods used are given in detail in the text.

	CONTROL			+ CONCANAVALIN-A		
Metabolite	dpm/flask	Recovered dpm (excluding glucose) (%)	Specific activity (dpm/μg atom C)	dpm/flask	Recovered dpm (excluding glucose) (%)	Specific activity (dpm/μg atom C)
Glucose (initial)	4,633,180		51,864	4,633,180		51,864
Glucose (final)	3,756,760		48,237	3,137,260		45,280
Glucose (metabolised)	876,420			1,495,920		
Glycogen+oligo-saccharides	159,155	23.8		177,541	12.4	
Glutamate	62,669	9.3	14,882	99,308	6.9	22,537
Aspartate	32,616	4.9		41,501	2.9	
Lactate	178,179	26.5	54,297	700,470	48.9	44,540
CO_2	183,810	27.4	13.707	312,476	21.9	18,446
RNA	10,861	1.6		19,332	1.3	
DNA	10,300	1.5		12,940	0.9	
Protein	20,468	3.1		39,149	2.7	
Lipid	12,876	1.9		25,509	2.1	
Total dpm recovered	4,428,094			4,569,385		

2.4.2 Percentage contribution of glucose to respiratory fuel

The percentage contribution of glucose to the fuel of respiration in rat thymocytes can be calculated in three different ways: (i) by determining the dilution of the specific radioactivity of glucose carbon in $^{14}CO_2$; (ii) by assuming that the glucose carbon taken up and not found in lactate and pyruvate is completely oxidized to CO_2 by the tricarboxylic acid cycle; or (iii) by determining the net flux of glucose to CO_2 (from the percentage recovery of d.p.m.) as a percentage of the total CO_2 production. Method (ii) gives a substantial overestimate (in contrast to the case of spleen slices) because of the large component of the glucose uptake unaccounted for. Methods (i) and (iii) give similar values (estimate in Table 2.2: Method i). However, Suter and Weidemann (1975) have shown that the specific activities of CO_2, glutamate and aspartate are practically identical after incubation of spleen slices with [U-^{14}C]-glucose. It is suggested that these amino acids are labelled by isotope exchange through the glutamate and aspartate transaminase half-reactions (Haslam and Krebs, 1963; Krebs *et al.*, 1966). Puckle (1974) has shown that 14.2% of the d.p.m. from [U-^{14}C]-glucose may be recovered in labelled glutamate and aspartate produced by rat thymocytes and that the specific activity of the carbon incorporated into glutamate and CO_2 is very similar (Table 2.2). This loss to glutamate and aspartate is a major component of the labelled glucose not accounted for. If ^{14}C does enter glutamate and aspartate purely by isotope exchange, this is equivalent to loss of labelled oxidizable intermediates of the tricarboxylic acid cycle, and methods (i) and (iii) both underestimate the percentage contribution of glucose to the respiratory fuel. The following equation, which compares the specific activity of CO_2 (corrected for loss to glutamate and aspartate) with the specific activity of glucose carbon, was used to obtain estimations in Table 2.2:

Table 2.3

The effect of acetoacetate on [U-^{14}C]-glucose metabolism and the response to concanavalin-A in rat thymocytes

The values are taken from Hume *et al.* (1978)
Details of the method are given in the text and Table 2.1. Results are given as μmoles/10^{10} cells/3hr ± S.E.M. for 3 experiments. Glucose and acetoacetate were each added at a final concentration of 5mM.

SUBSTRATE ADDED	ACETOACETATE		GLUCOSE		GLUCOSE + ACETOACETATE	
± Concanavalin-A (50 μg/ml)	-	+	-	+	-	+
Oxygen uptake	391±18	400±22	360±12	391±12	414±20	421±25
Glucose uptake	-	-	55± 4	122± 4	45± 1	105± 7
Lactate Production	-	-	27± 3	134± 5	73± 3	172± 4
Pyruvate production	-	-	1.3±0.2	4.5±0.2	8.7±0.6	15.9±0.9
Acetoacetate uptake	84± 8	81± 7	-	-	91±13	81±14
β-Hydroxybutyrate production	20± 3	19± 1	-	-	26± 1	25± 1
Cpm in $^{14}CO_2$ (10^{-4})	-	-	6.3	10.0	1.5	2.8

Percentage contribution of glucose to respiratory fuel =

$$\frac{\text{d.p.m. in } {}^{14}CO_2 + 0.142 \text{ (d.p.m. lost from glucose)}}{\mu \text{ mols of } CO_2 \text{ produced}} \times \frac{6 \text{ (}\mu\text{moles of glucose added)}}{\text{d.p.m. in added glucose}} \times 100$$

This estimate (43%) suggests that, even in the presence of glucose, the percentage contribution of the endogenous respiration to the O_2 consumption remains at a high level (57%). This represents a 13-14% suppression of the endogenous metabolism by glucose, which may be attributed to the suppression of endogenous amino acid oxidation associated with the decrease in NH_4^+ production observed on addition of glucose (see above).

Acetoacetate (5mM) alone stimulated the basal rate of respiration of isolated thymocytes to the same extent as added glucose (Table 2.3). The net amount of acetoacetate consumed was sufficient to account for 65% of the cellular O_2 consumption when the production of 3-hydroxybutyrate was subtracted. When cells were incubated with [U-^{14}C]-glucose plus acetoacetate, acetoacetate was the preferred fuel of respiration. Acetoacetate greatly inhibited the flow of glucose carbon to $^{14}CO_2$ and 90% of the net glucose uptake in its presence could be accounted for by the production of lactate and pyruvate. Thus, acetoacetate also reduced substantially the percentage of glucose uptake not accounted for, which according to the data in Table 2.2, may be equated with an inhibition of glycogen synthesis.

2.4.3 Effect of concanavalin-A and A23187 on glucose metabolism by T-lymphocytes

During the transformation of thymus lymphocytes to lymphoblasts, their metabolic activity is altered in specific ways. Among the many early changes that occur, the enhanced uptake of metabolites (e.g., sugars and amino acids) and ions (e.g., Ca^{2+}, K^+) may be of importance in

Fig. 2.2 Effect of concanavalin-A and A23187 on the uptake of ^{45}Ca and 3-o-methyl glucose by rat thymus lymphocytes

The data on the rate of 3-o-methyl glucose uptake by rat thymus lymphocytes are taken from the B.Sc (honours) thesis of A.J. Laird (1974); and the data on the rate of ^{45}Ca-uptake was taken from L.M. Russell (unpublished observations). Details of the method are given in the text. Where appropriate, concanavalin-A (10µg/mg) and A23187 (0.4µg) were present in the incubation medium.

n moles
/10^8 cells
3-o-methyl glucose uptake
con A
A-23187
control
0
2
4
6
8
0
20
40
60
Time (sec)

n moles Ca2+/ 10^8 cells
A-23187
con A
control
0
10
20
30
40
0
10
20
30
Time (min)

initiating and regulating the altered pattern of glucose oxidation seen in stimulated lymphocytes.

For instance, Fig. 2.2 shows that, in isolated rat thymus lymphocytes, the addition of concanavalin-A causes a marked stimulation (about 2.5-fold) in the initial rate of 3-o-methyl glucose transport. The Ca^{2+}-ionophore A23187 causes an almost identical change in the transport rate when at least 50μM free Ca^{2+} is present in the incubation medium. These two observations suggest that enhanced glucose transport in stimulated lymphocytes depends on externally added Ca^{2+} although the basal rate of glucose uptake can be supported independently of external Ca^{2+}. Further experiments have shown (Fig. 2.2) that the initial rate of ^{45}Ca uptake increased (2.5-fold) during the first 10 minutes after the addition of concanavalin-A but underwent no further change after 30 minutes. On the other hand, addition of A23187 to an identical cell population increased ^{45}Ca uptake for a much longer period (L.M. Russell and M.J. Weidemann, unpublished observations).

As a consequence of the increased uptake of Ca^{2+}-ions and glucose following concanavalin-A addition, glucose utilization by thymus lymphocytes was doubled during a three hour incubation (Table 2.1). More than 73% of the additional glucose metabolised was accounted for by a disproportionally large increase in the production of lactate and pyruvate, with an associated increase in the lactate-pyruvate ratio (indicating a fall in the cytoplasmic NAD^+/NADH ratio, assuming that lactate dehydrogenase is in equilibrium). This led to a large increase in the percentage of the glucose taken up that could be accounted for purely by lactate and pyruvate production (i.e., from 38%-58%). Similar results have been observed after addition of phytohaemagglutinin to rat thymus lymphocytes (Culvenor and Weidemann, 1976).

The percentage of the glucose consumption not accounted for by lactate, pyruvate and CO_2 production decreased in the presence of concanavalin-A, but the absolute amount increased, suggesting that a 20-30% increase in glycogen synthesis may have occurred. The percentage contribution of glucose to the respiratory fuel also increased in response to concanavalin-A, irrespective of the method used to estimate it. Estimation in (Table 2.1) was calculated using equation (i) except that the percentage incorporation of counts from [U-^{14}C]-glucose into glutamate and aspartate was assumed to decline to the same extent as the percentage of glucose counts found in CO_2. Concanavalin-A reproducibly increased the percentage contribution of glucose to the respiratory fuel from 43% to 58%, which represents a 37% suppression of endogenous metabolism. It is not possible to decide from these results whether the effect of concanavalin-A was due simply to increased glucose metabolism or whether it also involved a specific inhibition of the utilisation of endogenous fuels. As seen in Table 2.1, the effect of A23187 on glucose oxidation by thymocytes was more variable than that of concanavalin-A. At the concentration used (0.4µg/ml) the addition of ionophore very closely mimicked the effect of concanavalin-A on all aspects of glucose metabolism; glucose uptake, lactate production and the percentage contribution of glucose to the fuel of respiration were all increased to approximately the same extent by both agents. In order to determine the relationship between the two responses we have titrated each agonist alone and each in the presence of a range of concentrations of the other. The results show that the effects of concanavalin-A and A23187 on glucose uptake and lactate production are partly additive. An example is given in Fig. 2.3, where lactate production in the presence and absence of concanavalin-A has been measured over a range of A23187 concentrations.

Fig.2.3 Effect of varying concentrations of A23187 on concanavalin-A stimulated CO_2 and lactate production

The data are taken from the collaborative Ph.D project work of D.A. Hume. Details of the method are given in Table 2.1, except that A23187 was added to give different concentrations in the medium as indicated in the Fig. Lactate and $^{14}CO_2$ were assayed as described in the text.

increment due to concanavalin-A addition

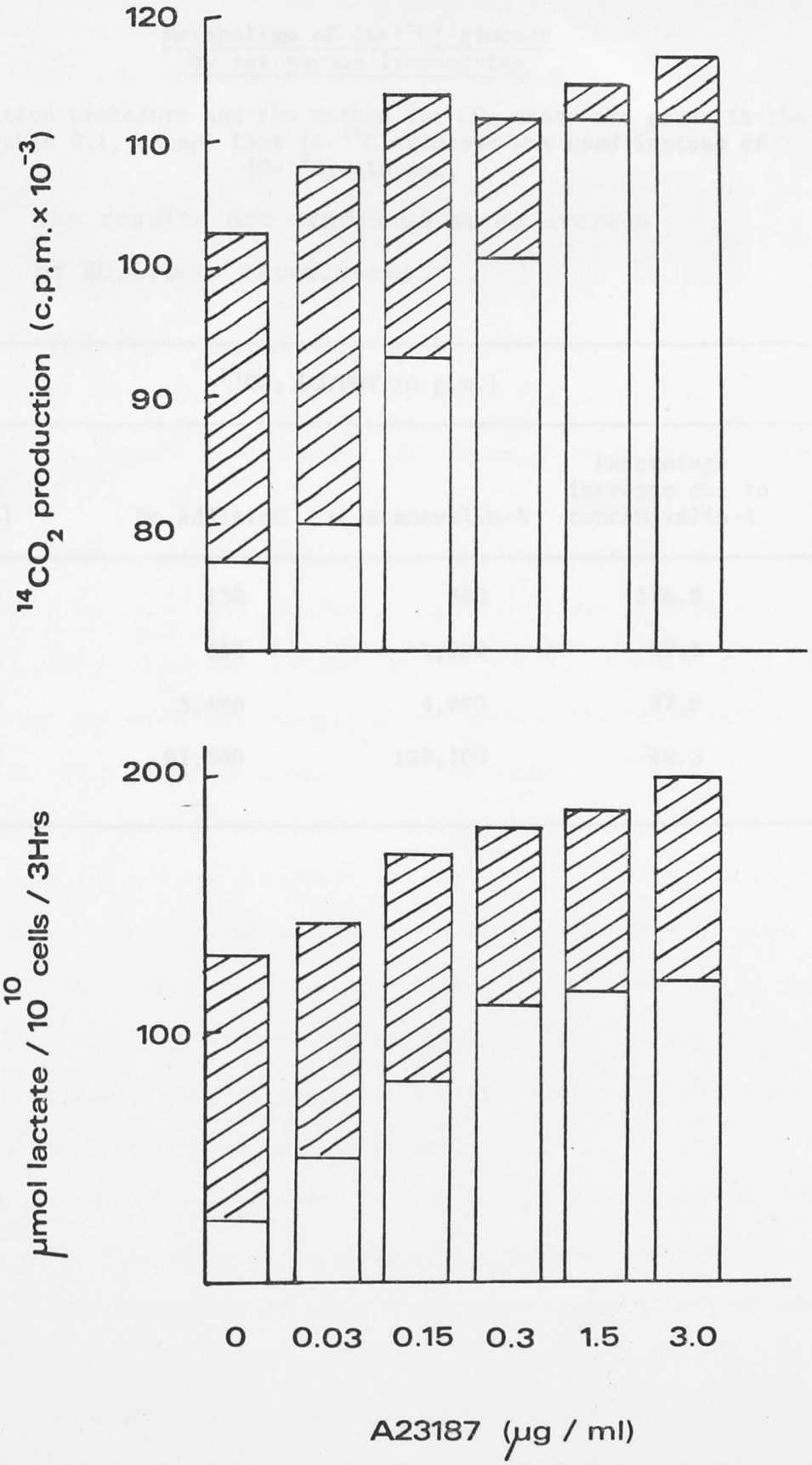

120
110
100
90
80
$^{14}CO_2$ production (c.p.m. x 10^{-3})
200
100
μmol lactate / 10^{10} cells / 3Hrs
0
0.03
0.15
0.3
1.5
3.0
A23187 (μg / ml)

Table 2.4

Metabolism of [6-^{14}C]-glucose by rat thymus lymphocytes

The incubation procedure and the method for CO_2 assay are given in the text and Table 2.1, except that [6-^{14}C]-glucose was used instead of [U-^{14}C]-glucose.

The results are expressed as an average of duplicate experiments.

	$^{14}CO_2$ OUTPUT (d.p.m.)		
Time (mins)	No addition	+Concanavalin-A	Percentage increase due to concanavalin-A
10	130	360	176.9
30	680	1,000	47.1
60	3,480	4,800	37.9
180	91,300	128,100	40.3

2.4.4 Effect of concanavalin-A and ionophore A23187 on $^{14}CO_2$ production from [U-^{14}C]- and [6-^{14}C]-glucose oxidation

Although addition of concanavalin-A or A23187 to thymocytes increased the production of lactate and pyruvate by 4-5-fold, only a 30-40% increase in the oxidation of [U-^{14}C]-glucose carbon to $^{14}CO_2$ was observed (Table 2.1). Experiments on isolated spleen cells gave essentially similar results, although the basal unstimulated glycolytic rate in these cells was much higher (Suter and Weidemann, 1975).

In order to establish that the increased CO_2 production by concanavalin-A may be due, at least in part, to increased oxidation of pyruvate, the $^{14}CO_2$ output from thymocytes oxidizing [6-^{14}C]-glucose was measured (Table 2.4). The respiratory $^{14}CO_2$ production was increased by concanavalin-A, both in short term incubations and at the end of 3 hours (Table 2.4), which rules out the possibility that an increased flux of glucose carbon through the oxidative segment of the pentose phosphate pathway may have been the main factor responsible for the increased $^{14}CO_2$ production (as it appears to be in phagocytosing macrophages and granulocytes). Furthermore, a low pentose phosphate pathway activity has been found in both unstimulated and mitogen-stimulated lymphocytes (Hedeskov, 1968) and in spleen slices (Suter and Weidemann, 1975). Thus, the weight of the available evidence suggests that the increase in $^{14}CO_2$ production in the presence of concanavalin-A is due to a specific increase in the oxidation of pyruvate generated by the glycolytic pathway.

The addition of acetoacetate as an alternative fuel did not affect the glycolytic stimulation induced by concanavalin-A (Table 2.3), but a much larger proportion of the glucose taken up was converted into lactate. The $^{14}CO_2$ production observed in the unstimulated cells was strongly inhibited by acetoacetate, but concanavalin-A was still able to stimulate this process. Together, these two observations indicate that concanavalin-A stimulates respiration by increasing [6-^{14}C]-glucose

oxidation at the reaction that produces acetyl-CoA from pyruvate rather than at the oxidation of acetyl-CoA (generated from a variety of sources such as acetoacetate, the oxidation of which is not stimulated by the mitogenic lectin) by the tricarboxylic acid cycle.

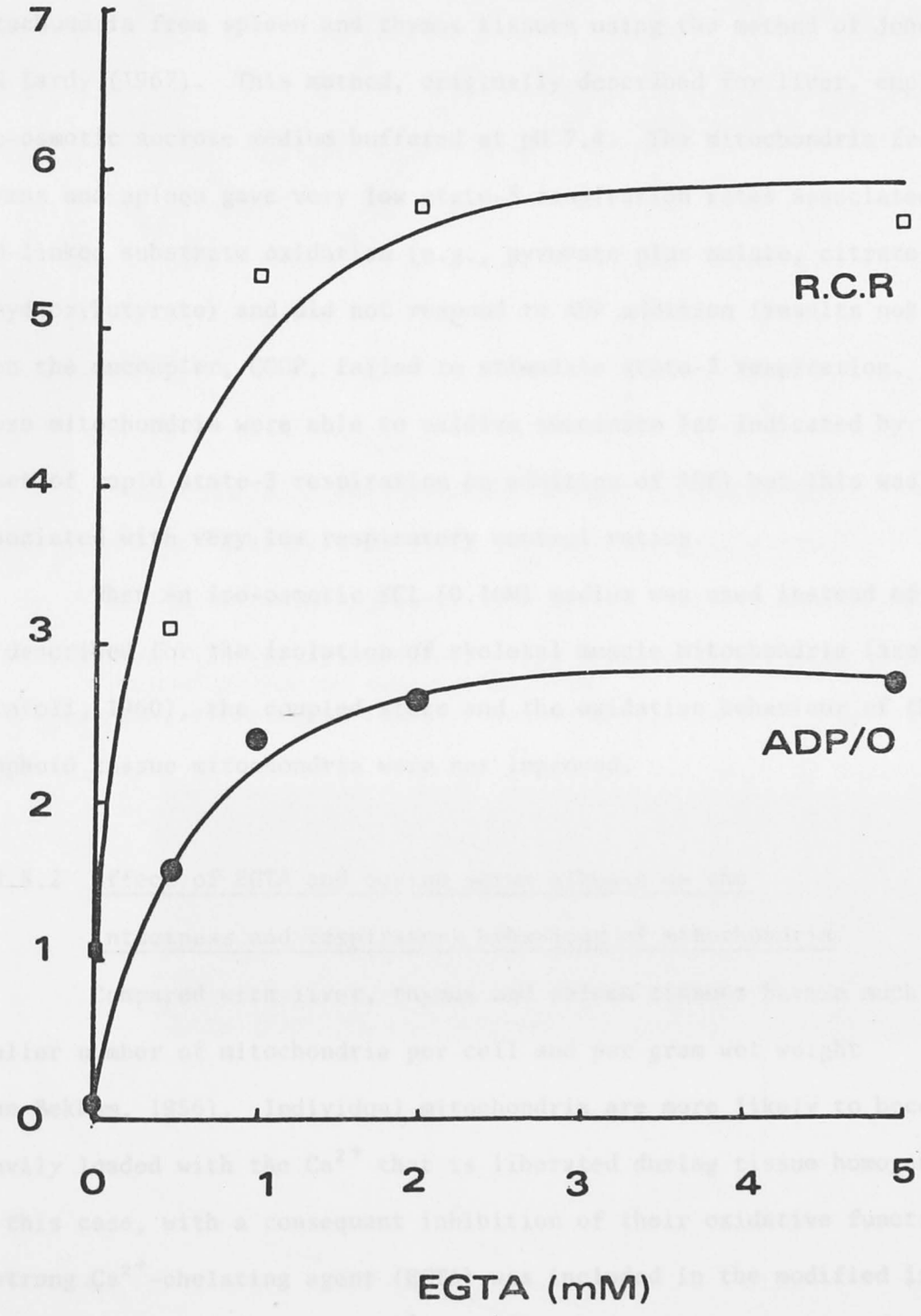
Earlier studies performed in this laboratory have indicated that pyruvate dehydrogenase is a low-activity, non-equilibrium, substrate-saturated and rate-limiting enzyme in lymphoid tissues (Suter, 1973; Suter and Weidemann, 1975) with a maximum catalytic capacity that is considerably slower than the rate of pyruvate production by the glycolytic pathway. Consequently, any increase in the rate of pyruvate oxidation would require the activation of mitochondrial pyruvate transport or pyruvate dehydrogenase by factors other than a simple increase in the substrate concentration. As Ca^{2+}-ions have been implicated in the activation of pyruvate dehydrogenase from various tissues (Denton *et al.*, 1972; Mukherjee and Jungas, 1975; Schuster and Olson, 1974), it is possible that the rise in cytoplasmic Ca^{2+} brought about by concanavalin-A (Parker, 1974; Freedman *et al.*, 1975) or A23187 (Luckasen *et al.*, 1974; Maino *et al.*, 1974) may be a sufficient stimulus to cause the increased $^{14}CO_2$ production from [6-^{14}C]-glucose that we have observed in whole cells.

In order to test this hypothesis, it was necessary to isolate intact and uncontaminated mitochondria from both spleen and thymus. Since relatively little attention has been paid in the past to the isolation and characterization of mitochondria from lymphoid tissues, some of their special properties have been studied prior to testing the effect of Ca^{2+} on pyruvate oxidation.

Fig.2.4 Effect of EGTA in the isolation medium on spleen mitochondrial oxidation

Rat spleen mitochondria were isolated as described in the text, except that EGTA was present at different concentrations in the isolation medium as given in the Fig. ADP/O and the respiratory control ratios (R.C.R.) were determined from state-3 and state-4 respiration rates as described in the text, using pyruvate (3mM) and malate (0.5mM) as the respiratory substrates.

The results represent an average of duplicate experiments.


7
6
5
4
3
2
1
0
R.C.R
ADP/O
0
1
2
3
4
5
EGTA (mM)

2.4.5 Isolation of mitochondria

2.4.5.1 Medium of choice for the isolation of mitochondria

Attempts were made initially to isolate intact, well-coupled mitochondria from spleen and thymus tissues using the method of Johnson and Lardy (1967). This method, originally described for liver, employs an iso-osmotic sucrose medium buffered at pH 7.4. The mitochondria from both thymus and spleen gave very low state-3 respiration rates associated with NAD-linked substrate oxidation (e.g., pyruvate plus malate, citrate and 3-hydroxybutyrate) and did not respond to ADP addition (results not shown). Even the uncoupler, CCCP, failed to stimulate state-3 respiration. However, these mitochondria were able to oxidize succinate (as indicated by the onset of rapid state-3 respiration on addition of ADP) but this was associated with very low respiratory control ratios.

When an iso-osmotic KCl (0.16M) medium was used instead of sucrose, as described for the isolation of skeletal muscle mitochondria (Azzone and Carafoli, 1960), the coupled state and the oxidative behaviour of the lymphoid tissue mitochondria were not improved.

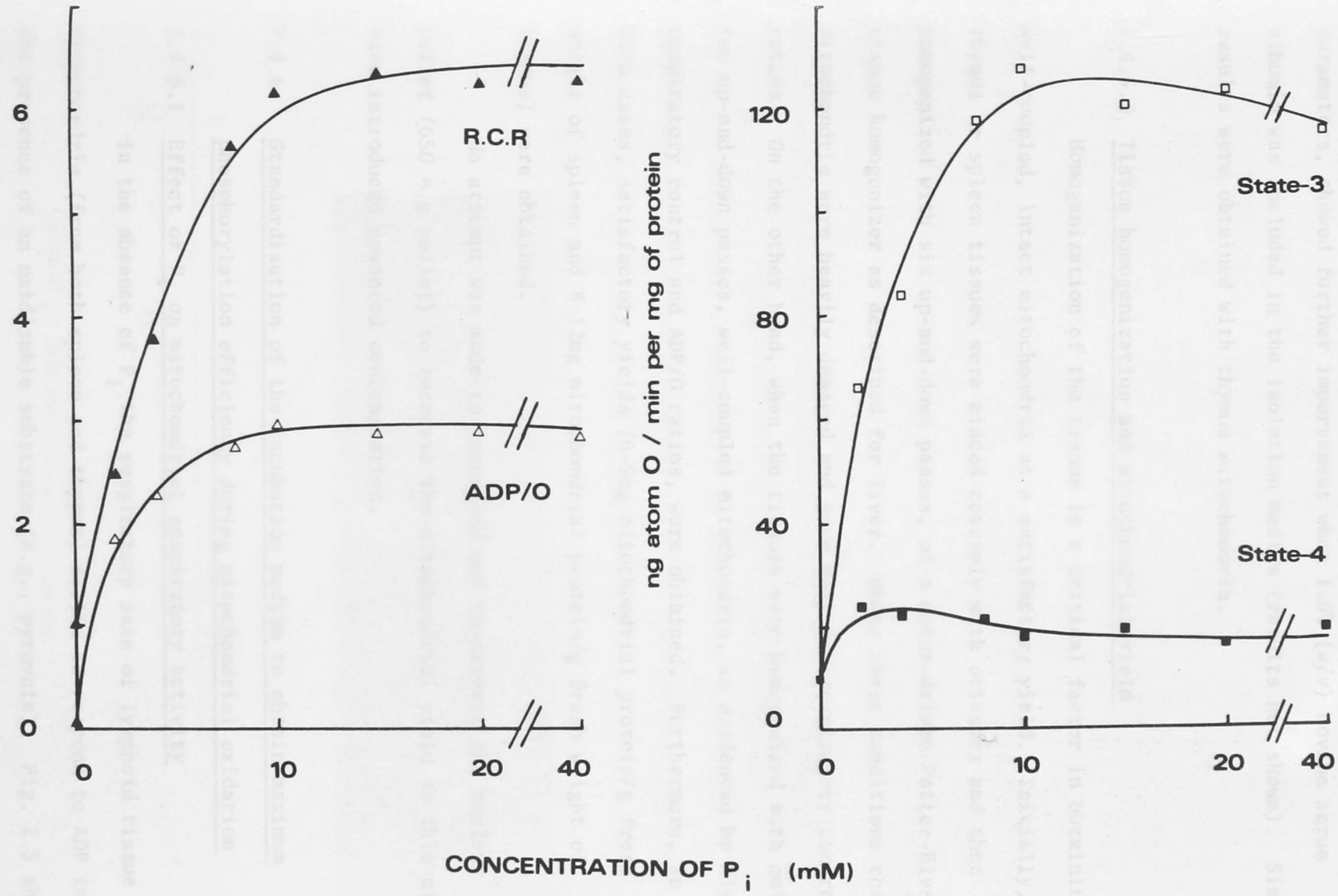
2.4.5.2 Effect of EGTA and bovine serum albumin on the intactness and respiratory behaviour of mitochondria

Compared with liver, thymus and spleen tissues have a much smaller number of mitochondria per cell and per gram wet weight (van Bekkum, 1956). Individual mitochondria are more likely to become heavily loaded with the Ca^{2+} that is liberated during tissue homogenization in this case, with a consequent inhibition of their oxidative functions. A strong Ca^{2+}-chelating agent (EGTA) was included in the modified isolation medium to prevent excessive Ca^{2+} loading during homogenization. Fig. 2.4 shows that the addition of at least 1.0mM EGTA was essential to obtain well-coupled spleen mitochondria. With 5.0mM EGTA in the isolation medium, the mitochondria gave high respiratory control and ADP/O ratios.

Fig.2.5 Effect of P_i on spleen mitochondrial oxidation

Details of measurement of mitochondrial respiration are given in the Methods section, except that varying concentrations of P_i were added to give the final concentrations shown in the Fig. ADP/O, respiratory control ratios, state-3 and state-4 respiratory rates were determined as described in the text.

The results represent an average of duplicate experiments.


6
4
2
0
R.C.R
ADP/O
0
10
20
40
120
80
40
0
State-3
State-4
0
10
20
40
ng atom O / min per mg of protein
CONCENTRATION OF P_i (mM)

The quality of the mitochondria, as assessed by these two parameters, showed further imporvement when 1.0% (w/v) bovine serum albumin was included in the isolation medium (results not shown). Similar results were obtained with thymus mitochondria.

2.4.5.3 Tissue homogenization and mitochondrial yield

Homogenization of the tissue is a critical factor in obtaining well-coupled, intact mitochondria at a satisfactory yield. Initially, thymus or spleen tissues were minced coarsely with scissors and then homogenized with six up-and-down passes, of a motor-driven Potter-Elvehjem tissue homogenizer as described for liver. Under these conditions the mitochondria were heavily damaged and gave very low respiratory control ratios. On the other hand, when the tissues were homogenized with only two up-and-down passes, well-coupled mitochondria, as evidenced by high respiratory control and ADP/O ratios, were obtained. Furthermore, in both cases, satisfactory yields (6-9mg mitochondrial protein/g fresh weight of spleen and 8-12mg mitochondrial protein/g fresh weight of thymus) were obtained.

No attempt was made to resuspend and re-extract the nuclear pellet (650 × g pellet) to increase the mitochondrial yield as this might have introduced unwanted contamination.

2.4.6 Standardisation of the incubation medium to obtain maximum phosphorylation efficiency during mitochondrial oxidation

2.4.6.1 Effect of P_i on mitochondrial respiratory activity

In the absence of P_i the respiratory rate of lymphoid tissue mitochondria (from both spleen and thymus) failed to respond to ADP in the presence of an oxidizable substrate (e.g., pyruvate). Fig. 2.5 shows that at least 10mM P_i must be present in the incubation medium to obtain

Fig.2.6 Effect of mitochondrial protein concentration on rat spleen and thymus mitochondrial oxidations

Details of measurement of mitochondrial respiration are as given in the text, except that different concentrations of mitochondrial protein were added to give the final concentrations shown in the Fig. State-3 and state-4 respiration rates were calculated as described in the text.

The results represent an average of duplicate experiments.

Fig. 2.6

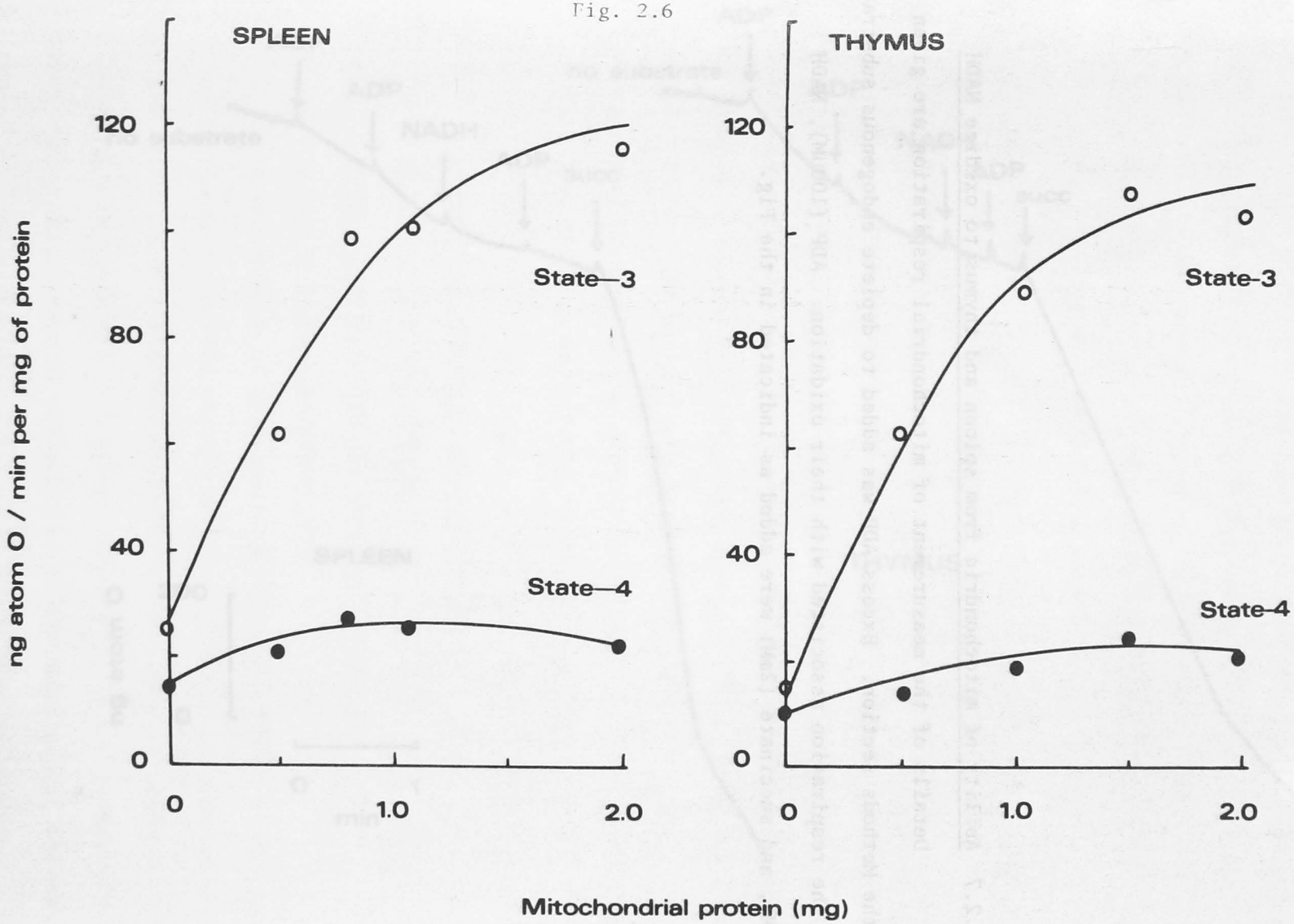
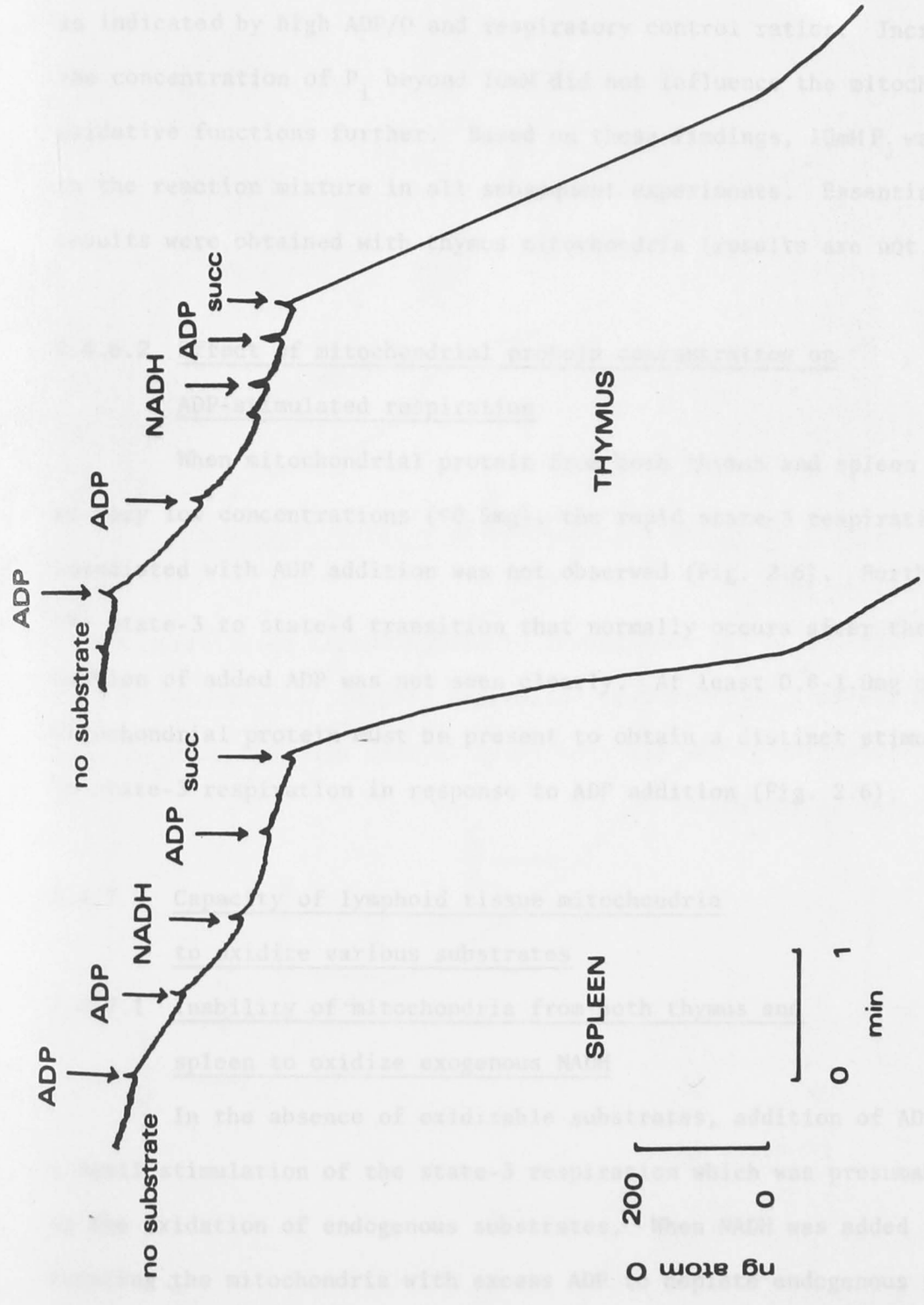

SPLEEN
THYMUS
120
80
40
0
State—3
State—4
State-3
State-4
0
1.0
2.0
ng atom O / min per mg of protein
Mitochondrial protein (mg)

Fig.2.7 Ability of mitochondria from spleen and thymus to oxidize NADH

Details of the measurement of mitochondrial respiration are given in the Methods section. Excess ADP was added to deplete endogenous substrates and the respiration associated with their oxidation. ADP (100μM), NADH (1mM), and succinate (2mM) were added as indicated in the Fig.


no substrate
ADP
ADP
NADH
ADP succ
no substrate
ADP
ADP
NADH
ADP succ
THYMUS
SPLEEN
ng atom O
200
0
0
1
min

the maximum response to ADP addition when using spleen mitochondria. Mitochondrial substrate oxidation is then well-coupled to ATP synthesis as indicated by high ADP/O and respiratory control ratios. Increasing the concentration of P_i beyond 10mM did not influence the mitochondrial oxidative functions further. Based on these findings, 10mM P_i was used in the reaction mixture in all subsequent experiments. Essentially similar results were obtained with thymus mitochondria (results are not shown).

2.4.6.2 Effect of mitochondrial protein concentration on ADP-stimulated respiration

When mitochondrial protein from both thymus and spleen was added at very low concentrations (<0.5mg), the rapid state-3 respiration normally associated with ADP addition was not observed (Fig. 2.6). Furthermore, the state-3 to state-4 transition that normally occurs after the exhaustion of added ADP was not seen clearly. At least 0.8-1.0mg of mitochondrial protein must be present to obtain a distinct stimulation in state-3 respiration in response to ADP addition (Fig. 2.6).

2.4.7 Capacity of lymphoid tissue mitochondria to oxidize various substrates

2.4.7.1 Inability of mitochondria from both thymus and spleen to oxidize exogenous NADH

In the absence of oxidizable substrates, addition of ADP caused a small stimulation of the state-3 respiration which was presumably due to the oxidation of endogenous substrates. When NADH was added (after treating the mitochondria with excess ADP to deplete endogenous substrates) there was no stimulation of state-3 respiration which could, however, be induced with succinate (Fig. 2.7). The inability of mitochondria from both spleen and thymus to oxidize exogenous NADH resembles that of

Fig.2.8 Ability of spleen mitochondria to oxidise long-chain fatty acids

Details of the measurement of mitochondrial respiration are given in the text. ADP (100μM), palmitate (100μM), carnitine (2mM), palmityl-carnitine (50μM) and coenzyme-A (0.5mM) were added in sequence as indicated in the Fig.

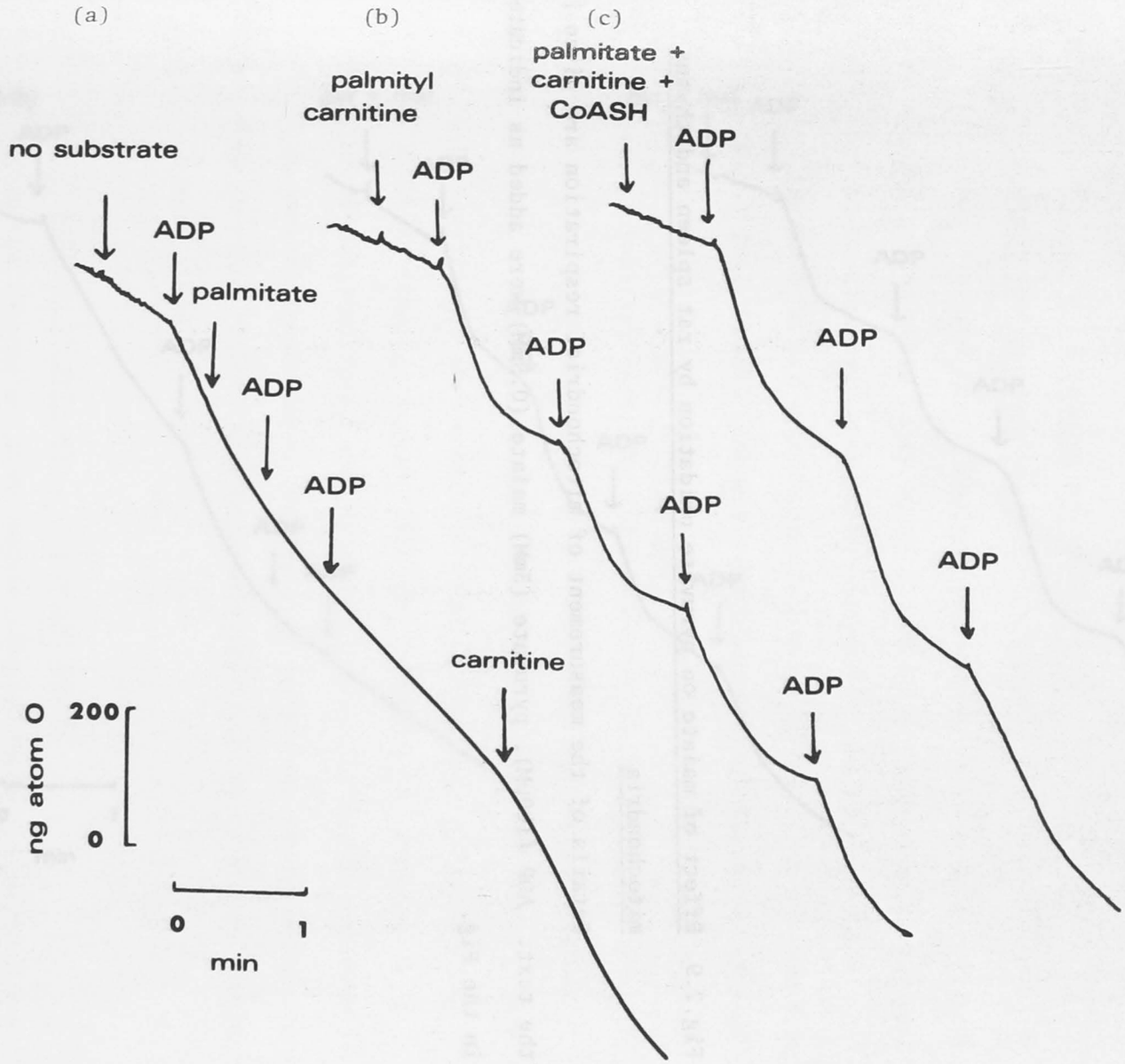
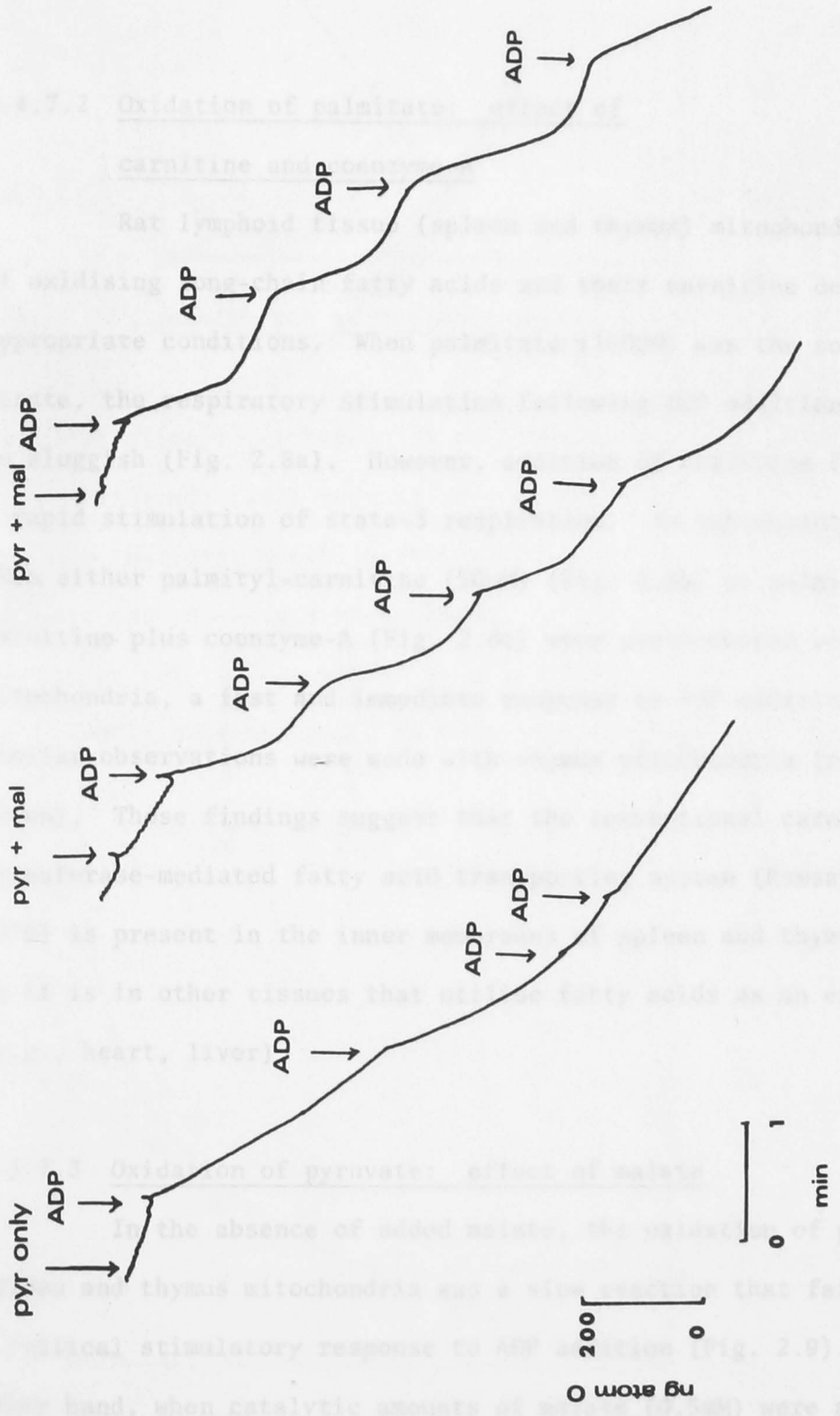

(a)
(b)
(c)
no substrate
palmityl carnitine
palmitate + carnitine + CoASH
ADP
palmitate
carnitine
ng atom O
200
0
0
1
min

Fig.2.9 Effect of malate on pyruvate oxidation by rat spleen and thymus mitochondria

Details of the measurement of mitochondrial respiration are given in the text. ADP (100μM), pyruvate (3mM) malate (0.5mM) were added as indicated in the Fig.


pyr only
ADP
ADP
ADP
ADP
ADP
ADP
pyr + mal
ADP
ADP
ADP
ADP
ADP
pyr + mal
ADP
ADP
ADP
ADP
ADP
ng atom O
200
0
0
1
min

other mammalian mitochondria (except those from heart) which have a permeability barrier to pyridine nucleotides at the inner mitochondrial membranes.

2.4.7.2 Oxidation of palmitate: effect of carnitine and coenzyme-A

Rat lymphoid tissue (spleen and thymus) mitochondria are capable of oxidising long-chain fatty acids and their carnitine derivatives under appropriate conditions. When palmitate (100μM) was the sole added substrate, the respiratory stimulation following ADP addition was found to be sluggish (Fig. 2.8a). However, addition of carnitine (2mM) induced a rapid stimulation of state-3 respiration. In subsequent experiments when either palmityl-carnitine (50μM) (Fig. 2.8b) or palmitate plus carnitine plus coenzyme-A (Fig. 2.8c) were preincubated with spleen mitochondria, a fast and immediate response to ADP addition was observed. Similar observations were made with thymus mitochondria (results not shown). These findings suggest that the conventional carnitine acyltransferase-mediated fatty acid transporting system (Ramsay and Tubbs, 1976) is present in the inner membranes of spleen and thymus mitochondria as it is in other tissues that utilise fatty acids as an energy source (e.g., heart, liver).

2.4.7.3 Oxidation of pyruvate: effect of malate

In the absence of added malate, the oxidation of pyruvate by spleen and thymus mitochondria was a slow reaction that failed to give a cyclical stimulatory response to ADP addition (Fig. 2.9). On the other hand, when catalytic amounts of malate (0.5mM) were added, a reproducible cyclical stimulation of the state-3 respiration rate was observed (Fig. 2.9), indicating that a source of oxaloacetate is essential

Fig.2.10 K_m and V_{max} for pyruvate oxidation by rat spleen mitochondria

Details of the measurement of mitochondrial respiration are given in the text. Pyruvate was added to give various final concentrations in the medium as shown in the Fig. State-3 respiration rates are plotted against pyruvate concentrations as indicated in Fig.——a; the same values are plotted in the double-reciprocal plot in Fig.——b. The values of K_m and V_{max} were calculated as described by Dixon and Webb (1964).

given
in
d
alues
f

Table 2.5

Ability of mitochondria from spleen and thymus to oxidize various substrates

Mitochondrial respiration was measured as described in the text.
Substrates were added to give final concentrations as indicated in the Table.
RCR and ADP/O ratios are calculated as described in the text.

	SPLEEN				THYMUS			
			ng atom O/min/mg				ng atom O/min/mg	
Substrates	ADP/O	RCR	State-3	State-4	ADP/O	RCR	State-3	State-4
Pyruvate (3mM) plus malate (0.5mM)	2.89	5.96	62.1	10.42	2.8	5.01	52.1	10.39
α-oxo glutarate (3mM) plus malate (0.5mM)	2.8	6.1	68.4	11.04	2.6	4.03	49.6	12.3
Succinate (2mM)	1.81	5.9	91.5	15.5	1.7	3.8	80.76	21.2
Malate (3mM)	2.81	3.3	50.6	15.4	2.4	3.3	36.3	11.2
Glutamate (3mM)	2.91	7.6	85.9	11.4	-	-	-	-
Citrate (2.5mM)	2.8	4.5	88.8	19.9	2.5	2.7	46.4	17.7
Palmityl-carnitine (50μM)	2.6	5.8	82.4	13.8	-	-	-	-
Fumarate (3mM)	2.3	3.9	48.6	12.6	2.41	3.61	41.8	11.6
Ascorbate (5mM) plus TMPD (1mM)	0.81	2.6	68.9	26.5	0.61	2.69	64.2	23.8
Ascorbate (5mM)	0.70	2.1	29.6	11.4	0.52	2.07	21.4	10.33

Fig.2.11 Effect of free Mg^{2+} (150μM) on spleen and thymus mitochondrial oxidation

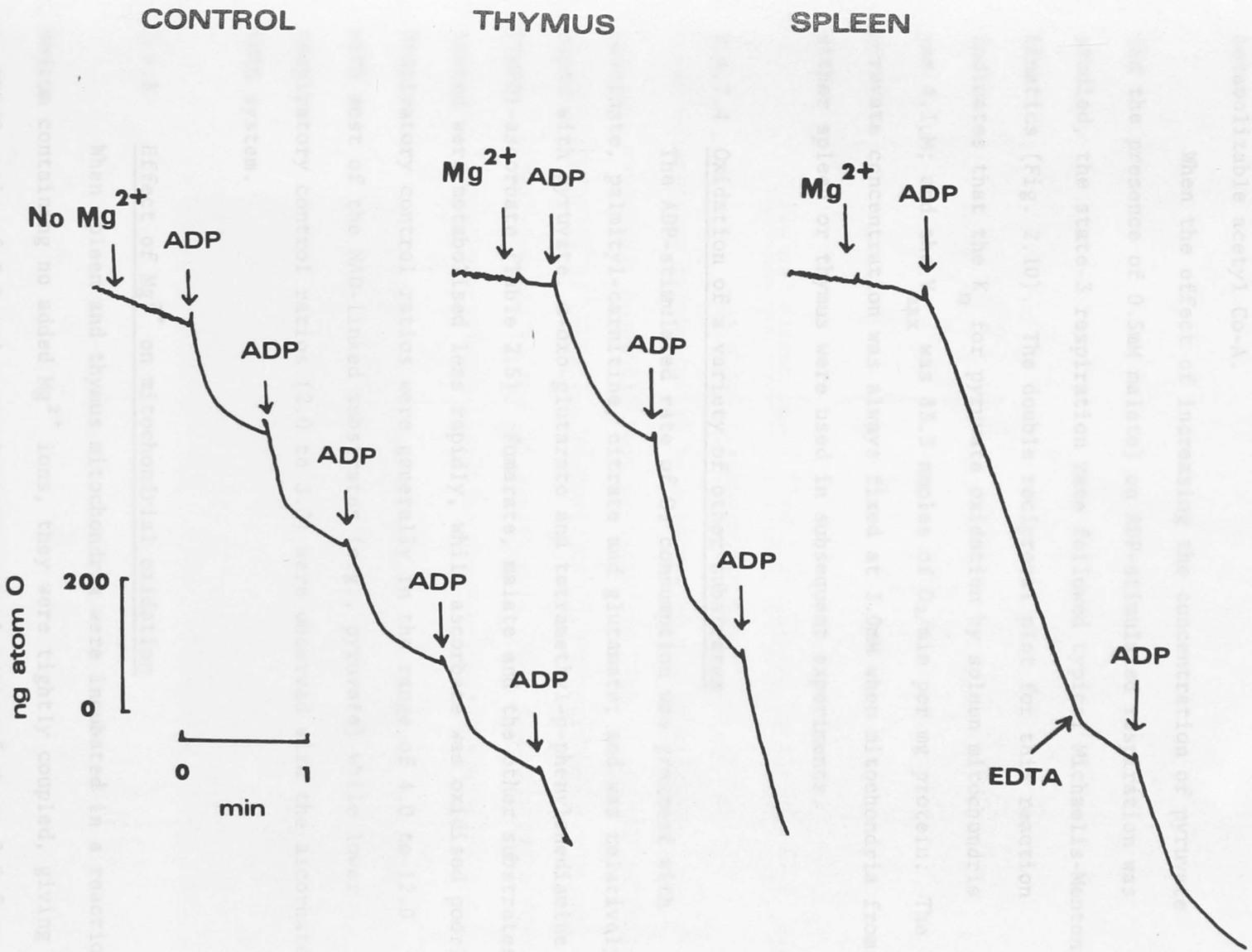
The incubation medium used is described in the Method section. Mg^{2+} was added to give a final free concentration of 150μM as indicated in the Fig. ADP (100μM), and EDTA (600μM) were added as indicated in the Fig.

control experiment refers to either thymus or spleen.

SPLEEN

THYMUS

CONTROL


CONTROL
THYMUS
SPLEEN
No Mg2+
Mg2+
Mg2+
ADP
ADP
ADP
ADP
ADP
ADP
ADP
ADP
ADP
ADP
EDTA
ng atom O
200
0
0
1
min

to promote the complete oxidation of pyruvate and to prevent the sequestration of the catalytic quantities of coenzyme-A present as non-metabolizable acetyl Co-A.

When the effect of increasing the concentration of pyruvate (in the presence of 0.5mM malate) on ADP-stimulated respiration was studied, the state-3 respiration rate followed typical Michaelis-Menton kinetics (Fig. 2.10). The double reciprocal plot for this reaction indicates that the K_m for pyruvate oxidation by spleen mitochondria was 4.1μM; and the V_{max} was 83.3 nmoles of O_2/min per mg protein. The pyruvate concentration was always fixed at 3.0mM when mitochondria from either spleen or thymus were used in subsequent experiments.

2.4.7.4 Oxidation of a variety of other substrates

The ADP-stimulated rate of O_2 consumption was greatest with succinate, palmityl-carnitine, citrate and glutamate; and was relatively rapid with pyruvate, α-oxo-glutarate and tetramethyl-p-phenylenediamine (TMPD)-ascorbate (Table 2.5). Fumarate, malate and the other substrates tested were metabolised less rapidly, while ascorbate was oxidised poorly. Respiratory control ratios were generally in the range of 4.0 to 12.0 with most of the NAD-linked substrates (e.g., pyruvate) while lower respiratory control ratios (2.0 to 3.0) were observed with the ascorbate-TMPD system.

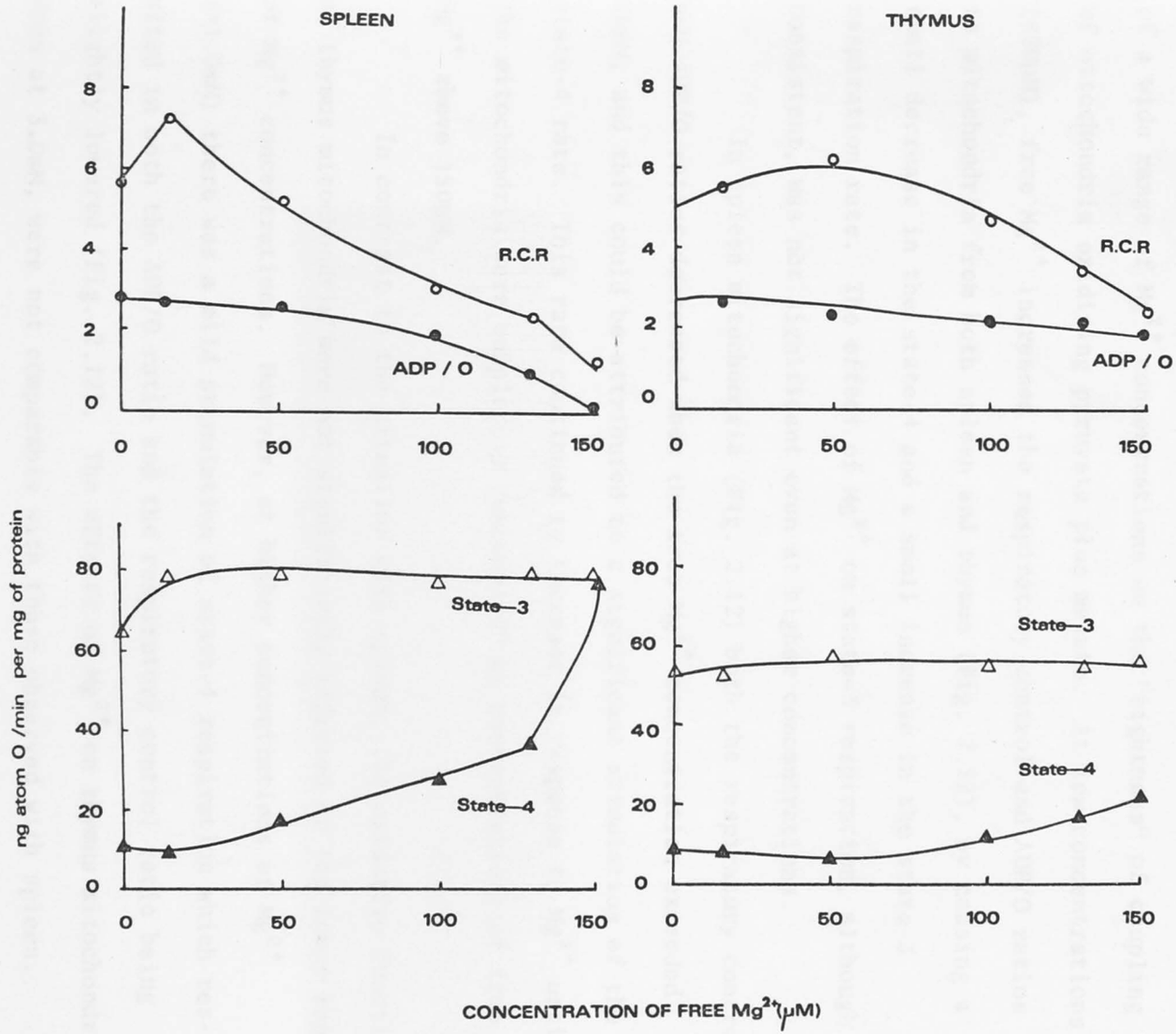
2.4.8 Effect of Mg^{2+} on mitochondrial oxidation

When spleen and thymus mitochondria were incubated in a reaction medium containing no added Mg^{2+} ions, they were tightly coupled, giving an ADP/O ratio of 2.9 and a respiratory control ratio of about 8.0 for pyruvate oxidation (Fig. 2.11a). In the case of spleen mitochondria, addition of free Mg^{2+} (>150μM) prolonged the state-3 respiration rate (Fig. 2.11b) which returned to state-4 only after the addition of excess

Fig. 2.12 Effect of varying free Mg^{2+} concentration on rat spleen and thymus mitochondrial oxidation

Details of the measurement of mitochondrial respiration are given in the text. Mg^{2+} was added to give different final free concentrations as shown in the Fig. ADP (100μM) was added to stimulate respiration using pyruvate (3mM) plus malate (0.5mM) as the substrates. State-3 and state-4 respiration rates and ADP/O and respiratory control ratios were calculated as described in the text.

The results represent an average of duplicate experiments.


SPLEEN
THYMUS
R.C.R
ADP / O
State—3
State—4
ng atom O /min per mg of protein
CONCENTRATION OF FREE $Mg^{2+}(\mu M)$

EDTA (600μM). EDTA also restored the cyclical response to ADP addition. In contrast, addition of the same amount of Mg^{2+} to thymus mitochondria under identical conditions did not change their respiratory status except that a small stimulation of the state-4 respiration rate occurred (Fig. 2.11c).

Further attempts were made to study systematically the effect of a wide range of Mg^{2+} concentrations on the "tightness" of coupling of mitochondria oxidising pyruvate plus malate. At low concentrations (<20μM), free Mg^{2+} increased the respiratory control and ADP/O ratios in mitochondria from both spleen and thymus (Fig. 2.12), by causing a small decrease in the state-4 and a small increase in the state-3 respiration rate. The effect of Mg^{2+} on state-3 respiration, although consistent, was not significant even at higher concentrations.

In spleen mitochondria (Fig. 2.12) both the respiratory control and ADP/O ratios decreased when the free Mg^{2+} concentration exceeded 20μM, and this could be attributed to a significant stimulation of the state-4 rate. This rate continued to increase in response to Mg^{2+} until the mitochondria were completely "uncoupled" at concentrations of free Mg^{2+} above 150μM.

In contrast to the situation with spleen, the oxidative functions of thymus mitochondria were not significantly affected in the lower range of Mg^{2+} concentrations. However, at higher concentrations of Mg^{2+} (>1.0mM) there was a mild stimulation of state-4 respiration which resulted in both the ADP/O ratio and the respiratory control ratio being slightly lowered (Fig. 2.12). The effects of Mg^{2+} on thymus mitochondria even at 3.0mM, were not comparable with those observed with spleen.

The contrasting responses of spleen and thymus mitochondria to Mg^{2+} addition suggests that there is a Mg^{2+}-dependent, ATPase-like activity that continuously regenerates ADP situated externally to the

Fig. 2.13 Effect of varying free Ca^{2+} concentration on rat spleen and thymus mitochondrial oxidation

Details of this experiment are similar to that described in Table 2.12, except that EGTA (1mM) was present in the incubation medium instead of EDTA (1mM), and Ca^{2+}, instead of Mg^{2+}, was added to give different final, free concentration in the medium, as given in the Fig.

The results represent an average of duplicate experiments.

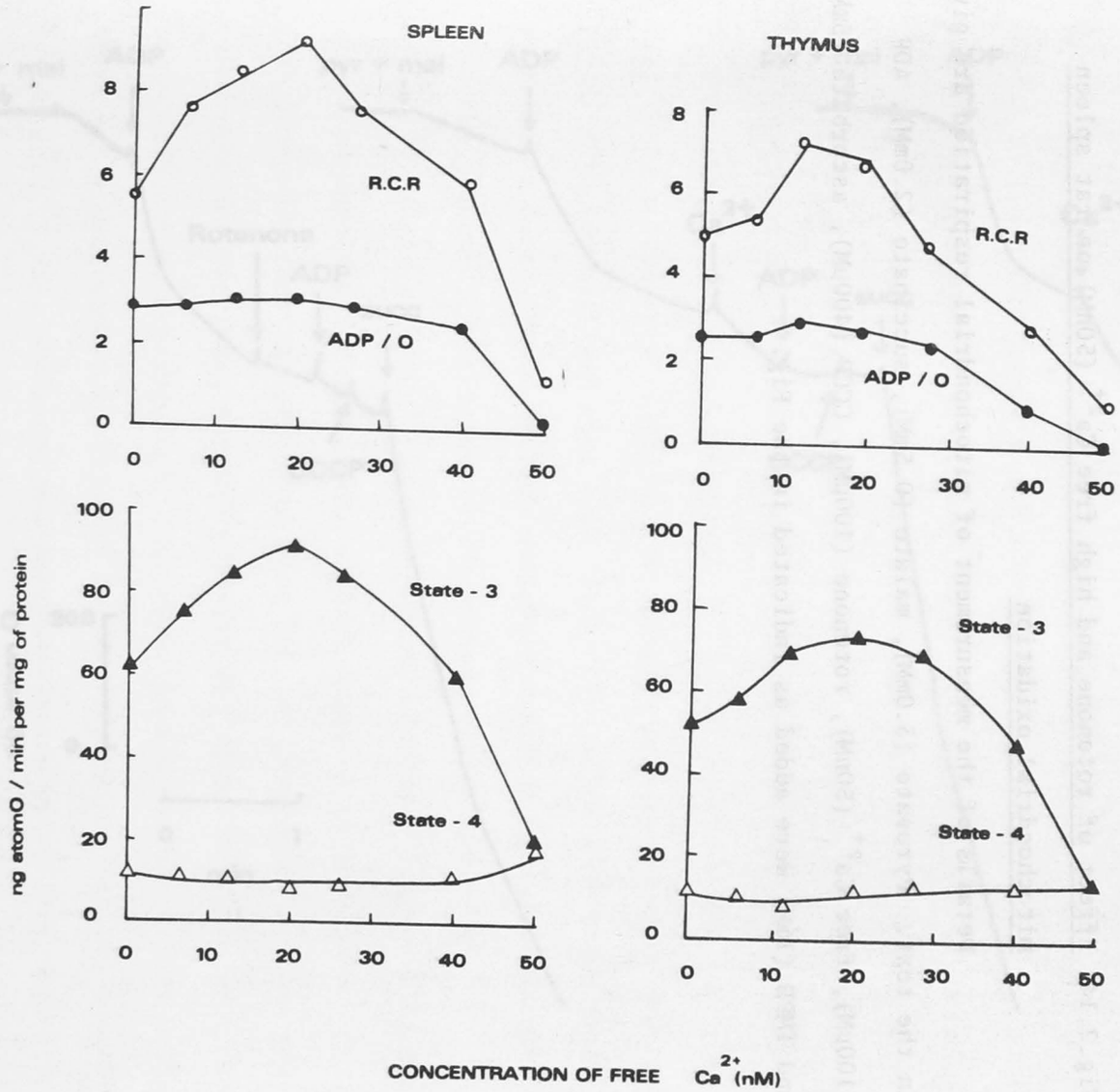
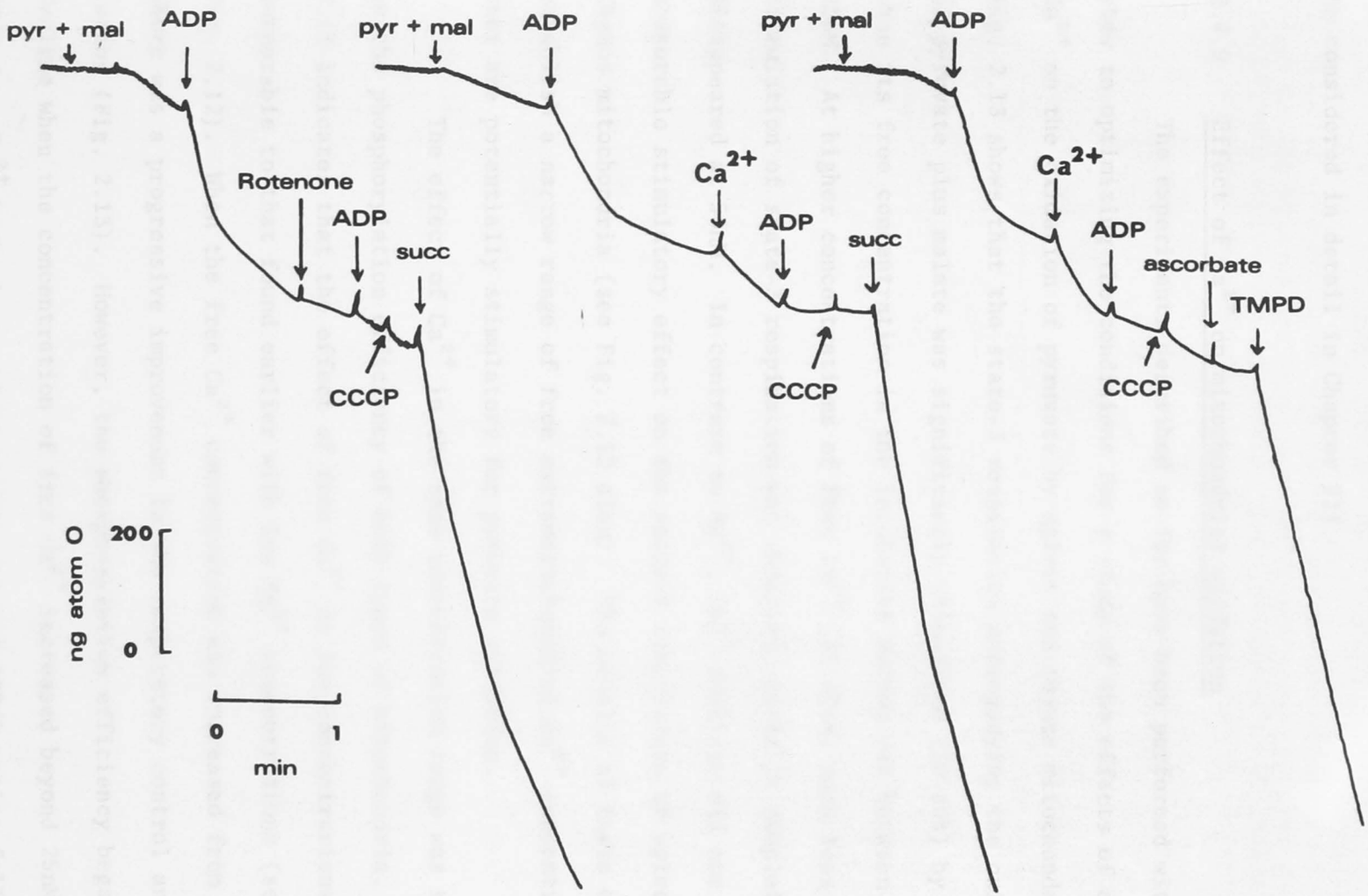

SPLEEN
THYMUS
R.C.R
ADP / O
State - 3
State - 4
ng atomO / min per mg of protein
CONCENTRATION OF FREE Ca2+ (nM)

Fig.2.14 Effect of rotenone and high free Ca^{2+} (50nM) on rat spleen mitochondrial oxidation

Details of the measurement of mitochondrial respiration are given in the text. Pyruvate (3.0mM), malate (0.5mM), succinate (2.0mM), ADP (100μM), free Ca^{2+} (50nM), rotenone (100μM), CCCP (400μM), ascorbate (5mM) and TMPD (1mM) were added as indicated in the Fig.


pyr + mal
ADP
Rotenone
ADP
succ
CCCP
pyr + mal
ADP
Ca2+
ADP
succ
CCCP
pyr + mal
ADP
Ca2+
ADP
ascorbate
TMPD
CCCP
ng atom O
200
0
0
1
min

EDTA-accessible space in intact spleen mitochondria that is absent from the thymus or present at only low activity. This question will be considered in detail in Chapter III.

2.4.9 Effect of Ca^{2+} on mitochondrial oxidation

The experiments described so far have been performed with a view to optimizing the conditions for a study of the effects of added Ca^{2+} on the oxidation of pyruvate by spleen and thymus mitochondria. Fig. 2.13 shows that the state-3 respiration accompanying the oxidation of pyruvate plus malate was significantly stimulated (30-40%) by Ca^{2+} when its free concentration in the incubation medium was between 0 and 25nM. At higher concentrations of free Ca^{2+} (25-40nM) much less stimulation of state-3 respiration was observed, until it completely disappeared at 50nM. In contrast to Mg^{2+}, Ca^{2+} addition did not have a measurable stimulatory effect on the state-4 respiration of spleen and thymus mitochondria (see Fig. 2.12 also). The results of these experiments establish a narrow range of free extramitochondrial Ca^{2+} concentrations that are potentially stimulatory for pyruvate oxidation.

The effect of Ca^{2+} in the same concentration range was studied on the phosphorylation efficiency of both types of mitochondria. Fig. 2.13 indicates that the effect of free Ca^{2+} at low concentrations was comparable to that found earlier with low Mg^{2+} concentrations (see also Fig. 2.12). When the free Ca^{2+} concentration was increased from 0-25nM, there was a progressive improvement in the respiratory control and ADP/O ratios (Fig. 2.13). However, the phosphorylation efficiency began to decline when the concentration of free Ca^{2+} increased beyond 25nM; at 50nM free Ca^{2+} both the respiratory control and ADP/O ratios fell dramatically to very low levels (Fig. 2.13).

Table 2.6

Effect of 20nM free Ca^{2+} on substrate oxidation by rat thymus and spleen mitochondria

Mitochondria were incubated and oxygen consumption was measured as described in the Methods section. Substrates were added to give the final concentrations given in this Table. Results represent the state-3 respiration rate in ng atoms oxygen/min/mg protein ± S.E.M. (3 experiments, where appropriate).

SUBSTRATES	SPLEEN	THYMUS
Pyruvate (3.0mM) } Malate (0.5mM)	62.2±8.2	52.1±4.6
+ Ca^{2+}	91.3±5.6	74.8±6.2
α-oxo glutarate (2.5mM)	68.4±4.9	49.6±3.9
+ Ca^{2+}	81.8±9.8	69.8±7.6
Succinate (2.5mM)	91.5±8.9	80.76
+ Ca^{2+}	95.2±6.7	87.22
Malate (3.0mM)	50.6	36.3
+ Ca^{2+}	48.3	41.2
Citrate (3.0mM)	88.8	46.4
+ Ca^{2+}	92.1	48.2
Glutamate (3.0mM)	85.9	-
+ Ca^{2+}	86.5	-
Palmityl Carnitine (50μM)	101.7	-
+ Ca^{2+}	98.3	-

When 600μM Ca^{2+} (free Ca^{2+} = 50nM) was added to either spleen or thymus mitochondria, it blocked the initiation by ADP of state-3 respiration associated with pyruvate plus malate oxidation and this effect could not be reversed by adding EDTA (Fig. 2.14). Under these conditions, the oxidation of other NAD-linked substrates (e.g., glutamate, 3-hydroxybutyrate) was also inhibited (results not shown); as the addition of an uncoupler (e.g., CCCP) failed to stimulate respiration (Fig. 2.14), the effect of Ca^{2+} on substrate oxidation is most likely to be due to the sequestering of free NADH to form a Ca^{2+}-NADH complex in the mitochondrial matrix (Chance, 1965; Vinogradov *et al.*, 1972). In contrast to the results with NAD-linked substrates, the addition of either succinate (Fig. 2.14) or ascorbate-TMPD (Fig. 2.14) was able to stimulate rapid state-3 respiration in the presence of 600μM Ca^{2+}. The similarity of the effect of adding high Ca^{2+} (Fig. 2.14) or rotenone (Fig. 2.14) on subsequent NAD-linked substrate oxidation gives additional support to the view that Ca^{2+} accumulation prevents the oxidation of NADH. These results indicate that concentrations of free Ca^{2+} in excess of 50nM are "toxic" to lymphoid tissue mitochondria at least with respect to the oxidation of NAD-linked substrates.

In order to test whether the stimulatory effect of low Ca^{2+} concentration (20nM) was specific for pyruvate oxidation, the oxidation of a number of other oxidizable substrates was studied. Table 2.6 shows that the oxidation of neither NAD-linked substrates (e.g., citrate, glutamate or malate) nor FAD-linked substrates (e.g., succinate) was stimulated by 20nM free Ca^{2+} in mitochondria from either spleen or thymus. On the other hand, the results show clearly a significant stimulation (30-40%) of the state-3 respiration rates associated with the oxidation of the two α-oxo acids, pyruvate (plus malate) and α-oxo-glutarate when mitochondria from either thymus or spleen were incubated with Ca^{2+}.

Fig. 2.15 Time-course of [I-^{14}C]-pyruvate oxidation by rat spleen mitochondria

Details of [I-^{14}C]-pyruvate oxidation (i.e., $^{14}CO_2$ collection) are as given in the text, except that no Ca^{2+} was present in the reaction mixture and the reaction was terminated at the time intervals, shown in the Fig. The results represent an average of duplicate experiments.

Fig. 2.15

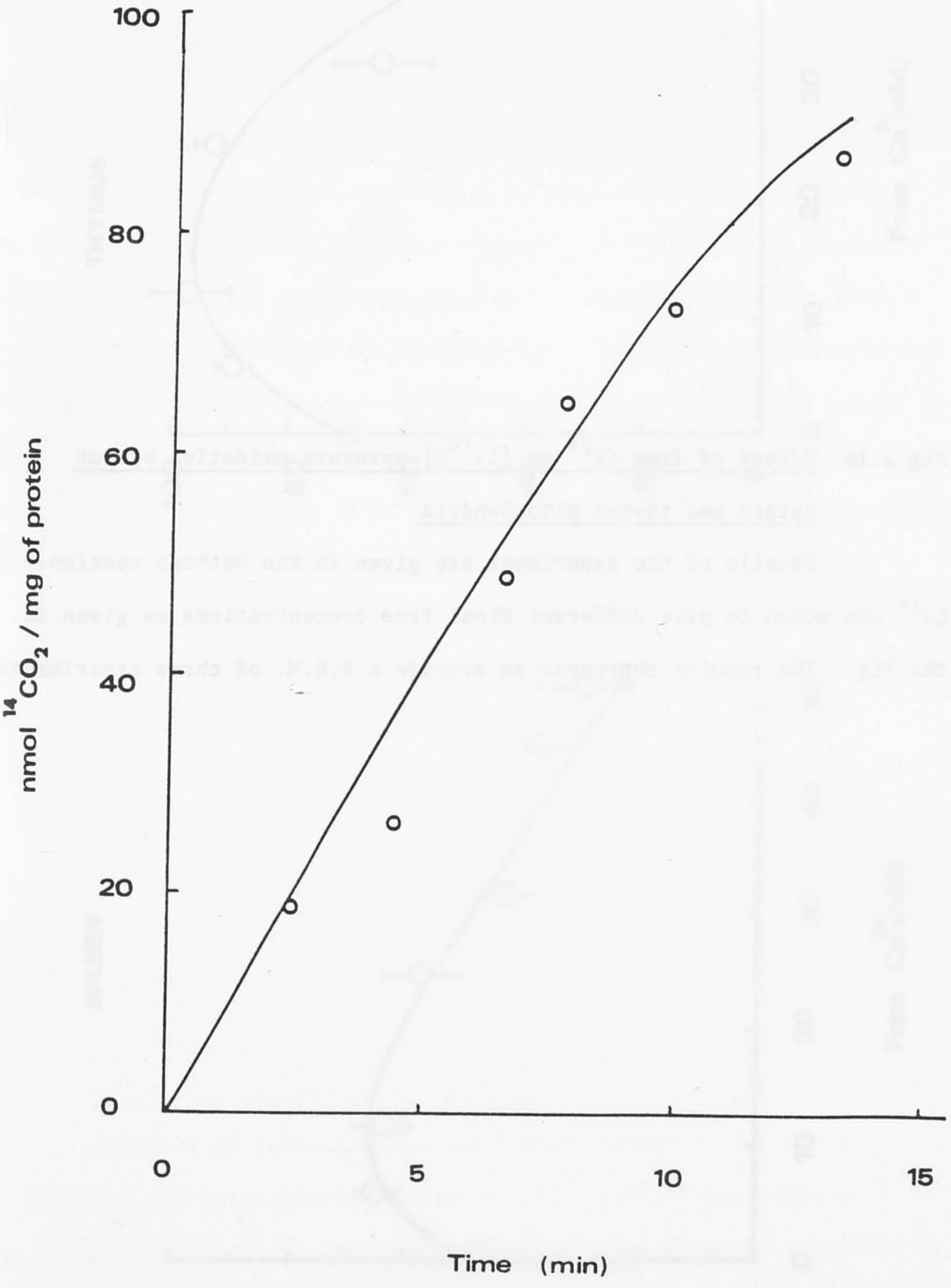
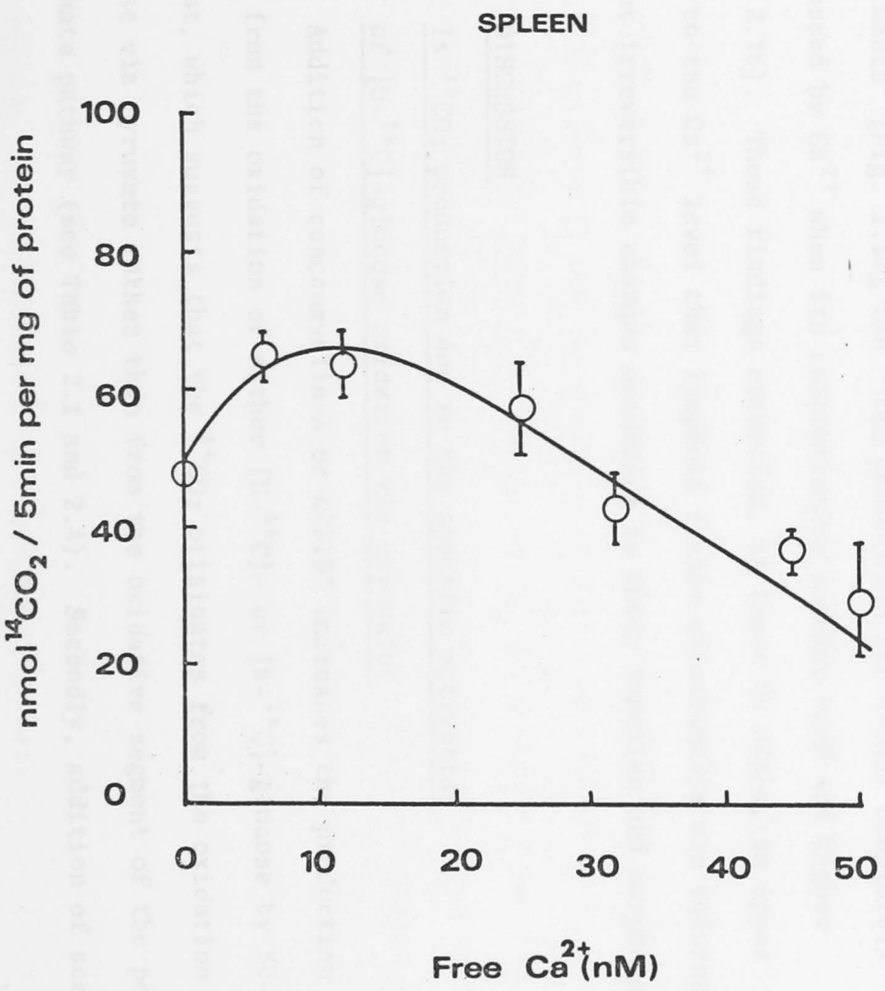
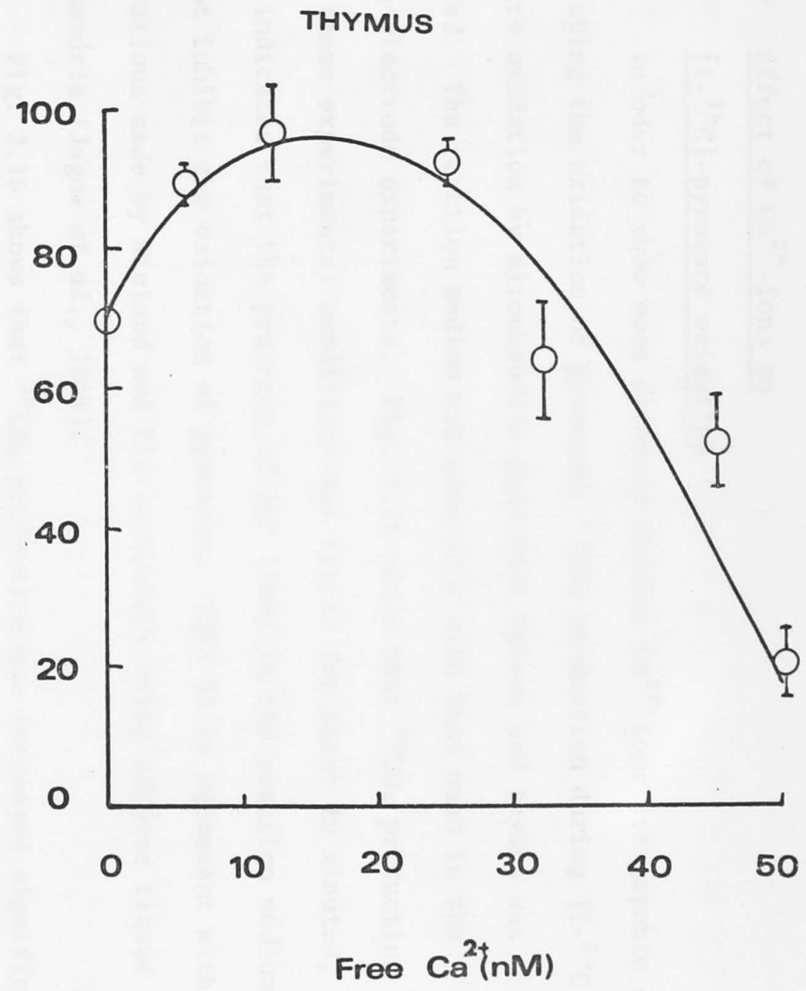


Fig.2.16 Effect of free Ca^{2+} on [1-^{14}C]-pyruvate oxidation by rat spleen and thymus mitochondria

Details of the experiment are given in the Methods section. Ca^{2+} was added to give different final free concentrations as given in the Fig. The results represent an average ± S.E.M. of three experiments.


SPLEEN
nmol $^{14}CO_2$ / 5min per mg of protein
100
80
60
40
20
0
0
10
20
30
40
50
Free Ca^{2+}(nM)


THYMUS
100
80
60
40
20
0
0
10
20
30
40
50
Free Ca^{2+}(nM)

2.4.10 Effect of Ca^{2+}-ions on [I-^{14}C]-pyruvate oxidation

In oder to show more directly whether Ca^{2+} ions are capable of stimulating the oxidation of pyruvate, $^{14}CO_2$ production during [I-^{14}C]-pyruvate oxidation by mitochondria from both spleen and thymus was measured. The reaction medium was identical with that used in the oxygen-electrode experiments. Fig. 2.15 shows that $^{14}CO_2$ production under these experimental conditions was linear for about 10 minutes, which indicates that the presence of ADP (5mM) in the reaction medium did not inhibit the oxidation of pyruvate. This is in agreement with observations made by Wieland and his co-workers using adipose tissue mitochondria (Jagow *et al.*, 1968).

Fig. 2.16 shows that $^{14}CO_2$ production was increased significantly (by 30-50%) in the presence of Ca^{2+} (0-40nM) when either spleen or thymus mitochondria were used. As observed with the oxygen-electrode experiments (Fig. 2.14), the $^{14}CO_2$ production was almost completely suppressed by Ca^{2+} when its concentration reached 50nM and higher (Fig. 2.16). These findings establish, at least *in vitro*, an upper limit to the Ca^{2+} level that lymphoid tissue mitochondria can tolerate without irreversible changes occurring to their function and morphology.

2.5 DISCUSSION

2.5.1 Is $^{14}CO_2$ production due to the specific activation of [U-^{14}C]-glucose oxidation via pyruvate?

Addition of concanavalin-A or A23187 increases the production of $^{14}CO_2$ from the oxidation of either [U-^{14}C]- or [6-^{14}C]-glucose by 30-40 percent, which suggests that the $^{14}CO_2$ originates from the oxidation of glucose via pyruvate rather than from the oxidative segment of the pentose phosphate pathway (see Table 2.1 and 2.4). Secondly, addition of aceto-

acetate does not affect the stimulation of glycolysis by concanavalin-A as shown by the large flux of glucose to lactate observed in its presence (see Table 2.3). On the other hand, acetoacetate strongly inhibits $^{14}CO_2$ production during [U-^{14}C]-glucose oxidation (Table 2.3) and is therefore, when the two substrates are oxidized together, the preferred fuel of respiration (Table 2.3). An increased steady-state concentration of mitochondrial acetyl-CoA associated with acetoacetate oxidation could, in principle, inhibit the oxidation of pyruvate, as acetyl-CoA is a potent end-product inhibitor of pyruvate dehydrogenase (Randle *et al.*, 1974; Severson *et al.*, 1977). These observations indicate that the increased production of $^{14}CO_2$ observed during concanavalin-A stimulation is due to the specific activation of [U-^{14}C]-glucose oxidation via pyruvate.

2.5.2 Factors possibly involved in the stimulation of the oxidation of pyruvate to CO_2

2.5.2.1 Effect of pyruvate concentration

The steady-state concentration of pyruvate, which has been estimated to be about 81±6 nmoles of intracellular water in the freeze-clamped spleen and 89±31 nmoles/ml of intracellular water in thymus (Suter, 1973; Hickman, 1974), is enhanced 4- to 5-fold during the oxidation of [U-^{14}C]-glucose (5.0mM) by cells which have been treated with concanavalin-A or A23187 (Table 2.1). Since the K_m for pyruvate oxidation by spleen mitochondria is only 4.1μM (see Fig 2.10), the prevailing metabolic steady-state concentration of pyruvate both before and after stimulation would be sufficient to saturate the enzyme that oxidises pyruvate to acetyl-CoA and CO_2 (i.e., pyruvate dehydrogenase). However, the oxidation of glucose carbon to CO_2 via pyruvate increased by only 30-40%, which suggests that the increase in pyruvate oxidation is dependent on factors other than a rise in substrate concentration. This observation is consistent with the view that pyruvate dehydrogenase is a low activity,

non-equilibrium enzyme in lymphoid tissues (Suter, 1973)

2.5.2.2 Effect of Ca^{2+}

Arguments for and against the involvement of Ca^{2+} in the transformation of lymphocytes have been presented in chapter 1. The results presented in Fig. 2.2 show that both concanavalin-A and A23187 increase the permeability of the plasma membrane to Ca^{2+}. Together, these observations support the view that a small increase in cytoplasmic Ca^{2+} is a signal for the early metabolic events associated with lymphocyte transformation (see also Freedman *et al.*, 1975; Yasmeen *et al.*, 1977; Jensen and Rasmussen, 1977; Whitesell *et al.*, 1977; Hesketh *et al.*, 1977). If Ca^{2+} does play a role in lymphocyte transformation, it is worth considering whether there is a direct causal relationship between the observed increase in Ca^{2+} uptake (Fig. 2.2) and the increase (30-40%) in the oxidation of glucose carbon to CO_2 (Table 2.1). Having established that $^{14}CO_2$ production from [U-^{14}C]-glucose occurs via pyruvate oxidation, it is possible that Ca^{2+} activates this process by: (i) stimulating pyruvate transport across the inner mitochondrial membrane; or (ii) by enhancing the activity of pyruvate dehydrogenase.

Recent observations by Foldes and Barritt (1977) indicate that the addition of extramitochondrial Ca^{2+} (50 nmoles/mg) is inhibitory for pyruvate translocation into (liver) mitochondria. The Ca^{2+} concentration used in their experiments, however, seems to be very high compared with estimates of cytoplasmic free Ca^{2+} which place the level in the range of 10^{-5} to 10^{-7}M (Naninga, 1961; Borle, 1967; Rasmussen and Nagata, 1970). Since only a small increase in total Ca^{2+} uptake (1.2 fold) is observed during lymphocyte stimulation (Fig. 2.2; see also Freedman *et al.*, 1975), it is unlikely that the extramitochondrial concentration of Ca^{2+} (i.e., cytoplasmic Ca^{2+}) rises to the extent used *in vitro* by Foldes and Barritt

(1977). Their observations do not exclude the possibility that low Ca^{2+} concentrations may stimulate the transport of pyruvate across the mitochondrial membrane.

The activity of mitochondrial pyruvate dehydrogenase is believed to be controlled by the interconversion of active (dephosphorylated) and inactive (phosphorylated) forms of the enzyme (Linn *et al.*, 1969a, b; Denton *et al.*, 1975). Studies on pyruvate dehydrogenase phosphate phosphatase have shown that Ca^{2+} may lower the K_m of the enzyme for its substrate, leading indirectly to the activation of pyruvate dehydrogenase (Denton *et al.*, 1975); or Ca^{2+} may increase the binding of the phosphatase to the pyruvate dehydrogenase complex and thereby increase the amount of the active (dephospho) form. An increase in pyruvate oxidation in response to added Ca^{2+} has been observed in isolated mitochondria from a number of tissues (Fisher *et al.*, 1973; Schuster and Olson, 1974; Foldes and Barritt, 1977), but there has been no previous indication of the extreme sensitivity of this process to low free Ca^{2+} in a Ca^{2+}-buffered system, as reported in the present work (Fig. 2.13).

The inhibitory effects of Ca^{2+} on the oxidation of NAD-linked substrates at concentrations above 50nM is very similar to that exerted by rotenone (Fig. 2.14), and may be related to the formation of Ca^{2+}-NADH complexes within the mitochondrial matrix (Chance, 1965). More importantly from a physiological point of view, the results suggest that there is an upper limit of 50nM for the free Ca^{2+} concentration in the cytosol, beyond which there is irreversible damage to mitochondrial function. The results are consistent with the proposal that concanavalin-A and A23187 activate cellular pyruvate oxidation by increasing the concentration of free Ca^{2+} in the cytosol.

2.5.3 Distinctive properties of rat spleen and thymus mitochondria

When mitochondria are isolated from rat thymus and spleen in a medium containing only sucrose (250mM) and Hepes buffer (10mM; pH 7.4), they fail to oxidise NAD^+-linked substrates (e.g., glutamate, pyruvate plus malate). However, when they are isolated in the same medium supplemented with EGTA, the mitochondria are well-coupled and capable of oxidising most of the added substrates (Table 2.5). The absolute requirement for EGTA seems to be a special property of only spleen and thymus tissues, since mitochondria from tissues like liver and heart, that are capable of oxidising most NAD-linked substrates, can be prepared in isotonic sucrose alone. The addition of 1mM EGTA simply improves the mitochondrial quality. The differential requirement for EGTA in the isolation media can be explained in the following way. Rat spleen and thymus tissues, which partly depend on glycolysis for their energy supply (see Suter and Weidemann, 1975; 1976) are characterised by a very low mitochondrial content per cell (van Bekkum, 1956) compared with highly-active tissues like heart (Munn, 1976). Since a large amount of membrane-bound Ca^{2+} is likely to be released during tissue homogenization, the few mitochondria that are liberated from the lymphoid tissues will become heavily loaded with Ca^{2+}-ions if a suitable Ca^{2+}-chelating agent is not present in the medium. Furthermore, these mitochondria, unlike those of liver, have a very low tolerance for Ca^{2+} especially during NAD^+-linked substrate oxidation (see Fig.2.14). Taken together, these two observations explain the absolute need for a suitable Ca^{2+}-chelating agent (e.g., EDTA or EGTA) in the isolation medium to obtain well-coupled, respiring mitochondria from lymphoid tissues.

The quality of these mitochondria, as defined by their high respiratory control ratios, was found to be improved when bovine serum

albumin (1% w/v) was included in the isolation medium. This may be due to the ability of bovine serum albumin to stabilize the mitochondrial membranes during homogenization and to bind the long-chain fatty acids released as a result of phospholipase activation.

Lymphoid tissue mitochondria appear to require an unusually high concentration of P_i in the incubation medium (about 7-10mM; see Fig. 2.4), unlike other tissue mitochondria (e.g., liver and heart) which have a K_m for P_i of 0.5mM. It has been suggested that high concentrations of P_i may stimulate ATP synthase activity (Ebel and Lardy, 1975; Mitchell and Moyle, 1969) and thus promote active oxidative phosphorylation. More simply, P_i could be required in the phosphorylation process itself, to form ATP from ADP, or in the dicarboxylate exchange reactions (e.g., malate - P_i exchange; McGivan and Klingenberg, 1971), but the exact reason is not clear. The other striking observation with lymphoid tissue mitochondria is the ability of bivalent metal ions, expecially Mg^{2+}, to interfere with the oxidative reactions themselves. This phenomenon, and its underlying basis, will be discussed in the following Chapter.

CHAPTER 3

LOCATION OF AN OLIGOMYCIN-INSENSITIVE AND MAGNESIUM PLUS CALCIUM ION-STIMULATED ADENOSINE TRIPHOSPHATASE IN RAT SPLEEN MITOCHONDRIA

CHAPTER 3

LOCATION OF AN OLIGOMYCIN-INSENSITIVE AND MAGNESIUM PLUS CALCIUM ION-STIMULATED ADENOSINE TRIPHOSPHATASE IN RAT SPLEEN MITOCHONDRIA

3.1 INTRODUCTION

The results presented in Chapter II indicate that mitochondria isolated from spleen and thymus have many common properties: a requirement for EGTA in the isolation medium; the preferential oxidation of particular substrates; and their response to metal ions such as Ca^{2+}. Nevertheless, a striking difference exists between them in the degree to which substrate oxidation and the phosphorylation of ADP are coupled in the presence of Mg^{2+}. Thymus mitochondria, for instance, resemble those of rat liver in the sense that state-4 respiration and the associated respiratory control ratios are hardly influenced by the addition of Mg^{2+} at low concentration (<150 μM) (see Chapter II, Fig. 2.11; also Chance and Williams, 1956). In contrast, the state-4 respiration of spleen mitochondria is highly sensitive to Mg^{2+} ions and is stimulated dramatically at free concentrations as low as 150 μM Mg^{2+} (see Fig 2.11); the respiratory control ratio falls to 1.0 under these conditions, indicating that the phosphorylation efficiency is very low (ADP/O $\sim$ 0). In this respect spleen mitochondria resemble those isolated from guinea pig heart (Cleland and Slater, 1953; Chance and Baltschfsky, 1958; Chao and Davis, 1972), rat kidney-cortex (Gmaj *et al.*, 1974) and rat cerebral-cortex (Sugano and Nagai, 1971). These findings suggest that there is a Mg^{2+}-stimulated ATP-hydrolyzing reaction in the EDTA-accessible space of rat spleen mitochondria that continuously regenerates ADP. The work described in this Chapter is aimed at defining the nature and submitochondrial location of this reaction.

The existence of several ATPases in whole mitochondria or in submitochondrial particles has been suggested by several workers on the basis

of indirect evidence such as differential sensitivity to inhibitors (Hemker, 1963). Ohnishi and Ohnishi (1962) have extracted an ATPase from rat liver mitochondria which resembles actomyosin and appears to be different from the Mg^{2+}-requiring and 2,4 dinitrophenol-stimulated ATPase that was semi-purified by Pullman *et al.* (1960). Furthermore, in rat liver mitochondria, Beyer (1960) has observed that at least two different ATPases are released after prolonged ultrasonic radiation.

There is no conclusive evidence at present for or against the presence of more than one type of ATPase in mammalian mitochondria. Two different ATPases from rat spleen mitochondria that can be distinguished clearly from one another on the basis of their sensitivity to inhibitors, their requirements for added bivalent metal ions and their localization within the mitochondria are described in the present chapter.

3.2 MATERIALS

$[^{32}P]P_i$ was from the Australian Atomic Energy Commission, Lucas Heights, N.S.W., Australia. Digitonin from Sigma Chemical Co., St. Louis, MO, U.S.A. was recrystallized from hot absolute ethanol, dried and ground to a fine powder. A stock solution 2% (w/v) was prepared by adding warm sucrose (0.25M) to the powdered digitonin; it was then cooled to 0°C before use. Oligomycin (15% oligomycin-A and 85% oligomycin B) was from Sigma. Atractyloside was a gift from Professor E.M. Klingenberg of the Institute for Physical Biochemistry, University of Munich, W. Germany. Ionophore A23187 was from Lilly Research Laboratories, Indianapolis, IN., U.S.A. $CaCl_2$ of the highest purity obtainable was from Orion Research Inc., MA, U.S.A. All other chemicals used were of analytical grade.

3.3 METHODS

3.3.1 Isolation of mitochondria

Mitochondria from rat spleen and thymus were isolated as described in Chapter II. Mitochondria from rat skeletal muscle were isolated in iso-osmotic KCl medium by the method of Azzone and Carafoli (1960); and mitochondria from other rat tissues were isolated in iso-osmotic sucrose by the following methods: liver and kidney (Johnson and Lardy, 1967); heart (Cleland and Slater, 1953); brain (Ozawa *et al.*, 1966); and lung (Fischer *et al.*, 1973).

3.3.2 Measurement of mitochondrial respiration

Mitochondrial respiration was measured with the Clarke-type oxygen electrode (Reed, 1972), as described in Chapter II. Mitochondrial protein was estimated as described in Chapter II.

3.3.3 Depletion of bivalent metal ions from rat lymphoid tissue mitochondria by treatment with ionophore A23187

Intact rat lymphoid tissue mitochondria (spleen, 4.0mg; thymus, 2.8mg) were depleted of endogenous bivalent metal ions by incubation in a medium containing sucrose (0.25M), Hepes buffer (20mM; adjusted with 2M KOH to give pH 7.4), EDTA (2 mM) and A23187 (0.5nmol/mg of mitochondrial protein) for 5 min at 0 ℃, in a final volume of 2.0ml, as described by Reed and Lardy (1972). At the end of the incubation period, the solution was centrifuged at 8500 × g for 15 minutes to sediment the mitochondrial pellet. The pellet was resuspended in 2.0ml of a solution containing sucrose (0.25M), bovine serum albumin (1% w/v) and Hepes buffer (20mM; pH 7.4).

3.3.4 Digitonin fractionation of mitochondria

Different fractions of spleen mitochondria (e.g., outer membranes,

inner membranes etc.) were prepared by the digitonin fractionation method described by Schnaitman and Greenawalt (1968) for rat liver mitochondria. The terminology developed by these authors to designate the individual fractions was followed. A stock digitonin solution (2%) was prepared just prior to use, as described in the Material section. Aliquots of ice-cold digitonin solution were added, with continuous gentle stirring, to spleen mitochondrial suspensions to give different final concentrations of digitonin (0.5 to 2.5mg per 10mg of mitochondrial protein as given in the legends to Fig. 3.5). The resulting suspensions were stirred gently, but intermittently, for 20 minutes at 0°C and then diluted with 3 vol. of ice-cold iso-osmotic sucrose solution buffered with Hepes (50mM; pH 7.4). The diluted suspensions were homogenized by hand with one up-and-down pass of a loosely fitting homogenizer and centrifuged at 8500 × g for 15 minutes. The clear supernatant was drawn off carefully with a Pasteur pipette, leaving behind the fluffy layer and a negligibly small volume of the supernatant. The pellet was not resuspended again to extract the residual outer membranes, as this procedure might have damaged the mitoplasts and contaminated the supernatant. The pellets and supernatants from this step are designated the "low-speed pellet" and "low-speed supernatant" respectively. The low-speed supernatant was fractionated further by centrifugation at 100,000 × g for 1 hour. The pellet and supernatant from this second centrifugation are designated the "high-speed pellet" and "high-speed supernatant".

3.3.5 Assay of enzyme activities

The following enzyme assays were carried out with a Zeiss PMQ II spectrophotometer equipped with a Beckman recorder. For all assays, the reactions were initiated by adding appropriate amounts of enzyme to cuvettes of 1cm light path containing a final assay volume of 3.1ml.

For each enzyme, saturating substrate concentrations were chosen to give maximum velocities, as described in the literature. The basic assay mixture contained the following ingredients:

(i) for the monoamine oxidase determination (marker enzyme for mitochondrial outer membranes, Tabor *et al.*, 1954), monitored at 250nm -

benzylamine-HCl (2.5mM) and phosphate buffer (K^+-salt; 50mM; pH 7.6);

(ii) for the glutamate dehydrogenase determination (marker enzyme for mitochondrial matrix, Schmidt, 1974), monitored at 340nm -

NADH (0.2mM), ADP (1mM), triethanolamine buffer (50mM; pH 8.0), EDTA (2.5mM), ammonium acetate (100mM), lactate dehydrogenase (2.I.U) and 2-oxoglutarate (7.0mM);

(iii) for the adenylate kinase determination (marker enzyme for the inter-membrane space, Bergmeyer *et al.*, 1974), monitored at 340nm-

glycyl-glycine buffer (70mM; pH 8.0), NADP (0.75mM), glucose (15mM), hexokinase (10 I.U.), glucose 6-phosphate dehydrogenase (0.4 I.U.), KCN (0.45mM), ADP (3.0mM), $MgCl_2$ (5.0mM);

(iv) for the creatine kinase determination (Forster *et al.*, 1974), monitored at 340nm -

imidazole buffer (100mM; pH 6.9), glucose (20mM), $MgCl_2$

(10mM), ADP (1.0mM), AMP (10mM), NADP (0.7mM), creatine phosphate (35mM), hexokinase (1I.U./ml), glucose 6-phosphate dehydrogenase (1I.U./ml), N-acetyl cysteine (10mM). Because adenylatekinase (EC 2.7.4.3) activity is found in the same mitochondrial sub-fraction that contains creatine kinase (EC 2.7.3.2), excess AMP (10mM) was added to inhibit this enzyme (Forster *et al.*, 1974). As AMP did not inhibit adenylate kinase activity completely, the absorbance was first measured in the absence of creatine phosphate (which specifically measures adenylate kinase activity in all mitochondrial subfractions) and, after 2 minutes, creatine phosphate was added to each reaction mixture to measure the additional activity due to creatine kinase;

(v) for cytochrome oxidase (mitochondrial inner membrane marker enzyme) determination the polarographic method of Schnaitman *et al.* (1967) was used. The reaction medium contained -

phosphate buffer (75mM; pH 7.2), cytochrome-c (30µM), sodium ascorbate (3.7mM) and N,N,N',N'-tetramethyl p-phenylene diamine (0.3mM) in a final volume of 2.0ml, at 23°C. The residual cytochrome oxidase activity measured after KCN addition was subtracted from the test values.

(vi) ATP-pyrophosphohydrolase (EC 3.6.1.8) activity was assayed at 37°C by the method of Libermann *et al.* (1967). The reaction medium contained -

Tris-HCl (50mM; pH 10.0), $MgCl_2$ (5mM) and ATP (4mM) in a final volume of 1.0ml;

(vii) inorganic pyrophosphatase was assayed at 37°C by the method of Lowry (1957) except that the P_i liberated was assayed as described by Dulley (1975). The reaction medium contained -

veronal buffer (20mM; pH 7.5), tetrapotassium pyrophosphate (2mM), BAL (British Anti Lewsite) (1.6mM) and $MgCl_2$ (4mM) in a final volume of 0.1ml;

(viii) 5'-nucleotidase was assayed at 37°C essentially as described by Ray (1970), except that the liberation of P_i, instead of AMP, was used as a measure of the activity of the enzyme. The reaction medium contained -

Tris-HCl (25mM; pH 7.5), 5'-AMP (5mM) and $MgCl_2$ (5mM) in a final volume of 1.0ml.

3.3.6 Analytical methods

Concentrations of the following substances were determined enzymatically with a Zeiss PMQ II spectrophotometer using published methods: ATP by the method of Lamprecht and Trautschold (1974); ADP and AMP by the method of Jaworek *et al.* (1974). P_i was assayed by the modified method of Fiske and Subbarow (1925) described by Dulley (1975).

3.3.7 Synthesis of [γ-^{32}P]ATP

[γ-^{32}P]ATP was synthesized by the modification of the method of Glynn and Chappell (1964) described by Post and Sen (1967). The synthesized radioactive ATP was checked for purity by the ascending chromato-

graphic method of Morrison (1968), using DEAE-cellulose paper (Whatmann DE-81) and 0.6M ammonium formate buffer (pH 3.1). The results indicated that only 0.67% P_i, 0.45% ADP and negligible amounts of AMP were present in the [γ-^{32}P]-ATP preparation.

3.3.8 Assay of ATPase activity

(a) Using intact mitochondria. The reaction medium contained sucrose (0.25 M), EGTA or EDTA (1.0mM), Hepes buffer (25mM; pH 7.4), $MgCl_2$ (0.9mM, giving a calculated concentration of free Mg^{2+} ions of approximately 150μM), unlabelled ATP (150μM) and [γ-^{32}P]-ATP (3×10^6 d.p.m.) in a final volume of 3.0ml. The reaction was started by the addition of mitochondria (0.2-0.5mg protein).

(b) Using the isolated outer membrane preparation. The reaction medium used was identical to (a) except that sucrose was replaced by water. The reaction was started by addition of the mitochondrial outer membrane preparation (40-60μg protein).

In each case samples (200μl) were withdrawn from the assay mixture at different time intervals (shown in the Legends) and placed in ice-cold $HClO_4$ (20μl of 1.0 M). The mixture was centrifuged (2 minutes at 5000 $\times$ g) in an Eppendorf centrifuge to sediment the precipitated protein and an aliquot (100μl) of clear supernatant was neutralised with KOH (10μl of 1.0 M). The labelled inorganic phosphate liberated was separated from unhydrolysed [γ-^{32}P] ATP chromatographically (Sweig and Sherma, 1972), using Whatman No.1 paper. The paper strips were cut into 6 equal pieces (3.0cm) and placed into vials containing 10ml of scintillation fluid {15g of Butyl-PBD [5-(4-biphenylyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole], dissolved in 1.5 litres toluene and 1.0 litre 2-methoxyethanol}. The radioactive phosphate was counted in a Beckman LS-350 liquid scintillation counter. The inorganic phosphate liberated was calculated from the ^{32}P peak counts.

Fig.3.1 Differentiation between oligomycin-sensitive and -insensitive ATPases of rat spleen mitochondria

ATPase activities were assayed by using intact mitochondria (0.4mg) in the incubation medium containing [γ-^{32}P]-ATP (specific radioactivity 10^6 d.p.m./μmol) as described in the Experimental section, under the following conditions: □ , mitochondria alone; △ , plus atractyloside (50μM); ▲ , plus oligomycin (2μg/mg); ● , plus $MgCl_2$ (0.9mM) and atractyloside (50μM); ○ , plus $MgCl_2$ (0.9mM) and oligomycin (2μg/mg); ■ , $MgCl_2$ (alone).

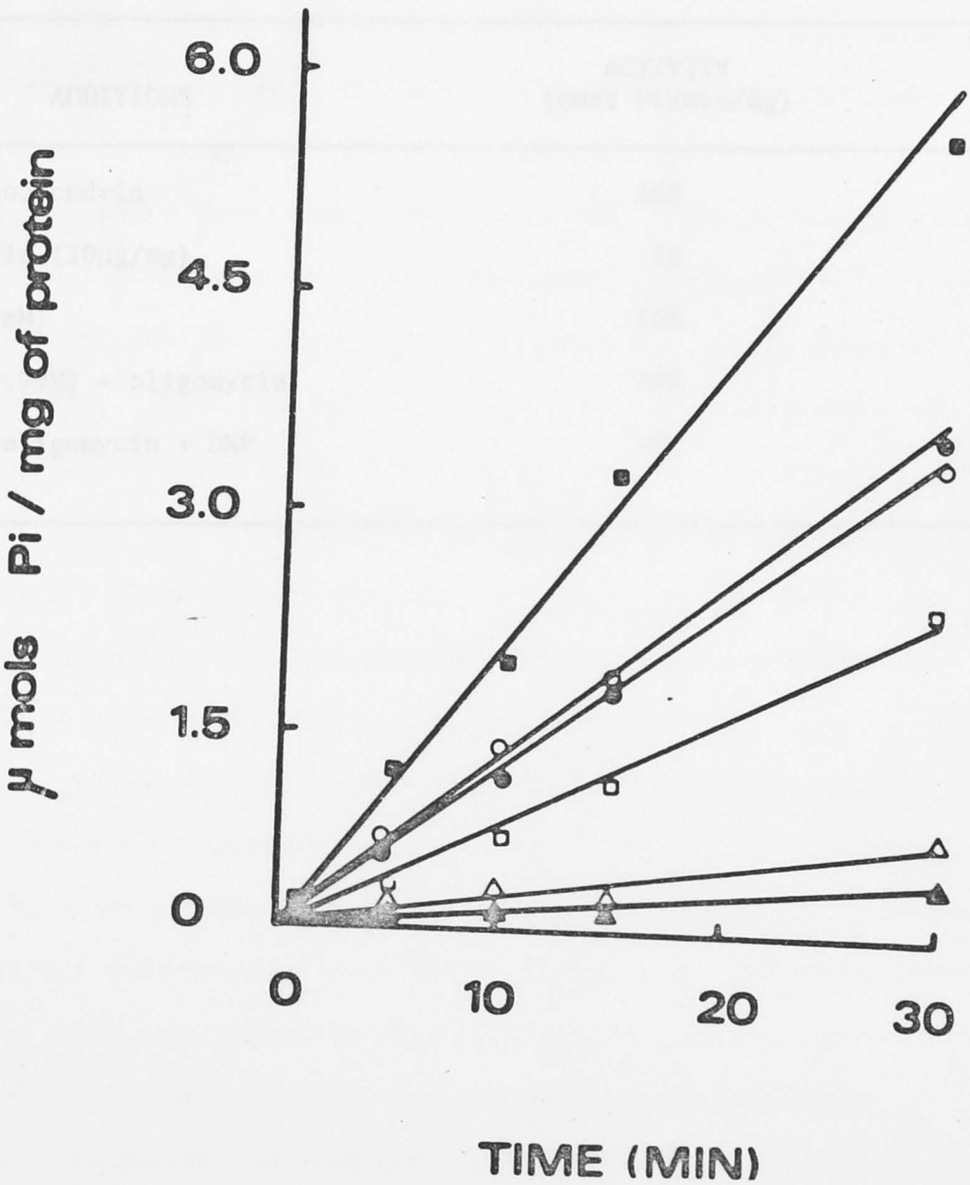

6.0
4.5
3.0
1.5
0
μ mols Pi / mg of protein
0
10
20
30
TIME (MIN)

Table 3.1

Effect of dinitrophenol (DNP) on rat spleen mitochondrial ATPase activities

The ATPase activity was assayed in intact mitochondria (0.4mg), at 22°C as described in the Experimental section. [γ-^{32}P]-ATP (specific activity 3.5×10^6 d.p.m./μ mol) was added to give a final concentration of 150μM in each case in a total assay volume of 3.0ml.

ADDITIONS	ACTIVITY (nmol Pi/min/mg)
Intact mitochondria	108
+ oligomycin (10μg/mg)	12
+ DNP (0.1mM)	148
+ $MgCl_2$ (0.9mM) + oligomycin	180
+ $MgCl_2$ + oligomycin + DNP	192

3.4 RESULTS

3.4.1 Differentiation between matrix (oligomycin-sensitive) and external (oligomycin-insensitive) ATPase activities from rat spleen and thymus mitochondria

The results of the polarographic studies described in Chapter II suggest that a Mg^{2+}-stimulated ATPase-like activity may be present in intact rat spleen mitochondria and absent from thymus. The release of ^{32}P from [γ-^{32}P]-ATP was used, therefore, as a more specific assay for the activity of this enzyme.

Fig. 3.1 shows that, even without added Mg^{2+}, spleen mitochondria possess a basal ATPase activity that is inhibited by about (85-90%) by oligomycin (2µg/mg) or atractyloside (50µM), and which represents the activity of the matrix ATPase. When free Mg^{2+} (150µM) was added to the incubation medium containing atractyloside or oligomycin (at the concentrations given above) there was a marked stimulation of the rate of ^{32}P liberation. This observation is consistent with the ability of free Mg^{2+}, over the same concentration range to stimulate the rate of state-4 respiration dramatically in these mitochondria (see Fig. 2.5).

The two ATPase activities observed can be distinguished clearly if their response to uncouplers (e.g., N,N'dinitrophenol) is considered. Using intact spleen mitochondria in the absence of added Mg^{2+}, the matrix space ATPase was stimulated by about 40% on addition of 0.1mM dinitrophenol (Table 3.1). This rate represents the maximum oligomycin-sensitive ATPase activity (148 nmols/min/mg) that has been observed in these experiments. Its specific activity is approximately equal to that of the Mg^{2+}-stimulated ATPase (168 nmols/min/mg), which, in contrast, is not significantly accelerated by dinitrophenol when measured in the presence of oligomycin (10µg/mg). The small stimulation observed (8-10%) was probably due to the release of a small fraction of the oligomycin-sensitive ATPase activity from the matrix during preparation. The additional ATPase

Fig.3.2 ATPases of rat thymus mitochondria

ATPase activities were assayed by using intact thymus mitochondria (0.3mg) in the medium containing [γ-^{32}P]-ATP (specific activity 10^6 d.p.m./µmol) as described in the Experimental section, under the following conditions ○, mitochondria alone; ● plus $MgCl_2$ (0.9mM); ▲, plus oligomycin (10µg/mg protein) and atractyloside (50µM).

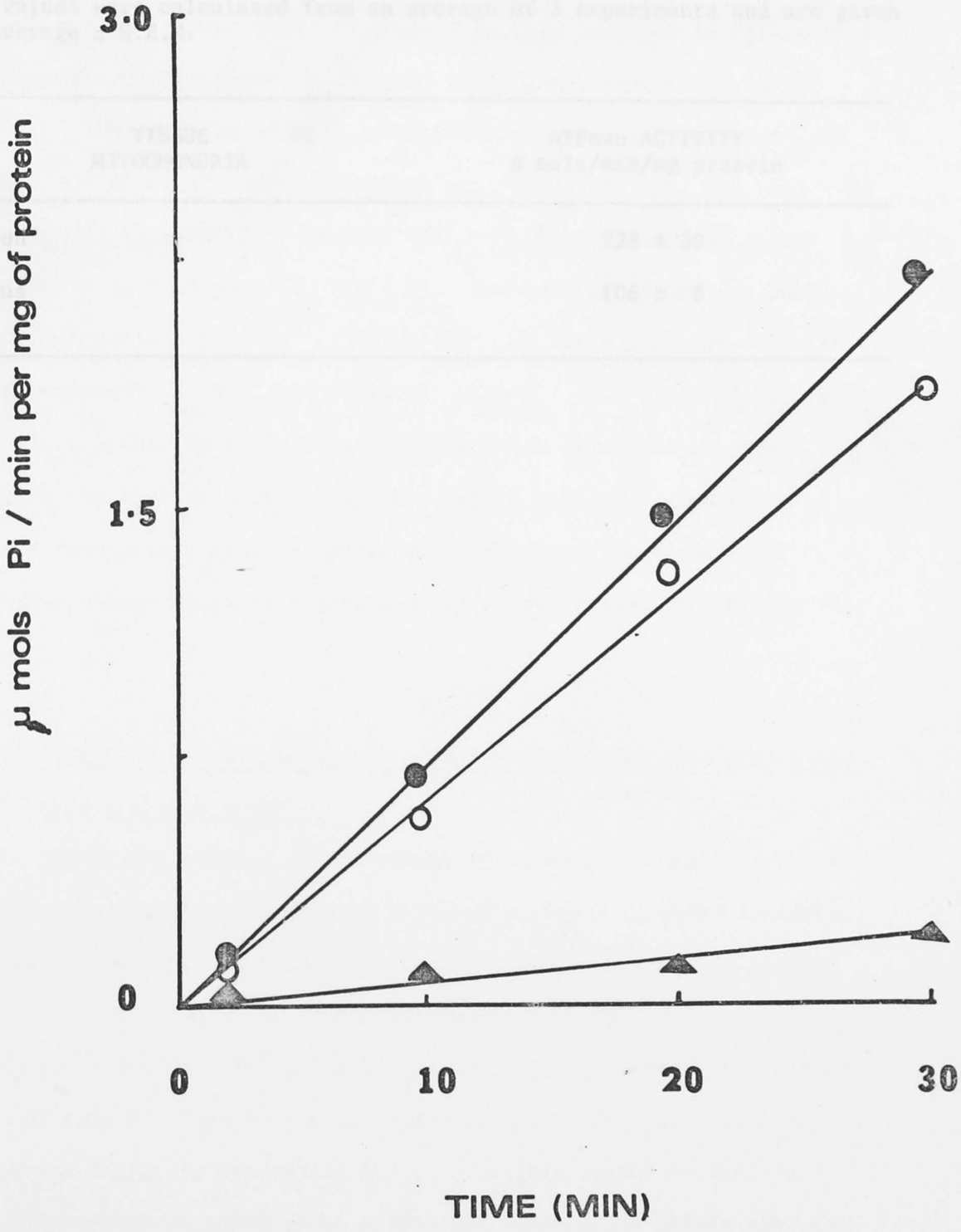

3·0
1·5
0
μ mols Pi / min per mg of protein
0
10
20
30
TIME (MIN)

Table 3.2

Mg^{2+}-stimulated ATPase activity of rat lymphoid tissue mitochondria

The ATPase activity was assayed using intact mitochondria from spleen (0.5mg protein) or thymus (0.5mg protein), at 30°C as described in the Experimental section. The reaction medium contained sucrose (250mM), Hepes (10mM; pH 7.4), ATP (2.0mM), [γ^{32}-P]ATP (3.5×10^6 d.p.m./µmol), $MgCl_2$ (5.0mM), atractyloside (50µM) and oligomycin (10µg/mg protein). The values were calculated from an average of 3 experiments and are given as average ± S.E.M.

TISSUE MITOCHONDRIA	ATPase ACTIVITY n mols/min/mg protein
spleen	228 ± 39
thymus	106 ± 8

activity accessible to [γ^{32}-P]-ATP added outside the atractyloside-barrier can thus be differentiated from the matrix space ATPase by virtue of its dependency on added Mg^{2+}-ions and its insensitivity to oligomycin and dinitrophenol.

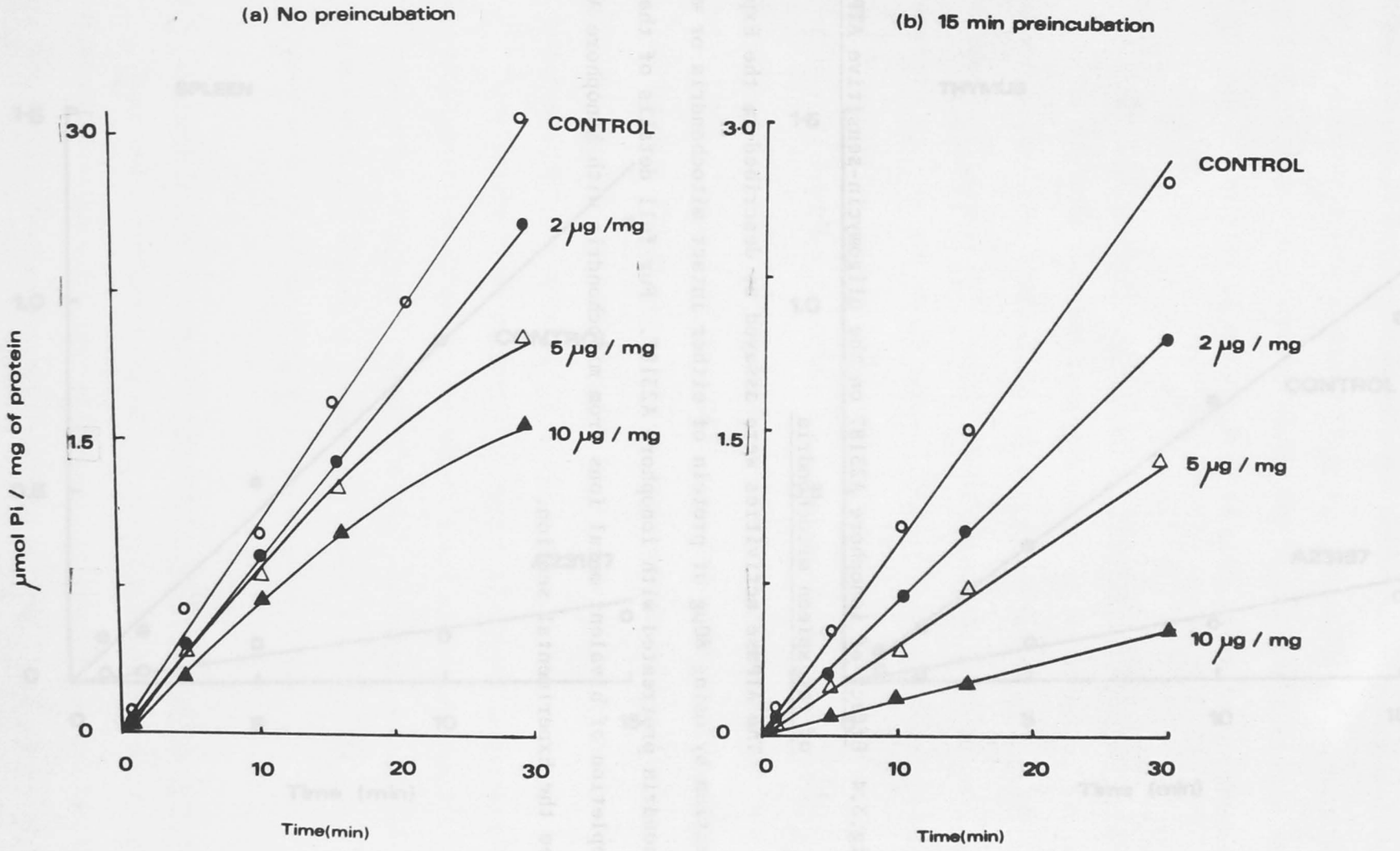
Fig.3.2 shows that, in the absence of added Mg^{2+}, thymus mitochondria possess a basal ATPase activity similar to that observed in spleen that is significantly inhibited by oligomycin (2µg/mg) and atractyloside (50µM). The addition of Mg^{2+} (150µM) did not have a significant additional effect on the measured ATPase activity (Fig.3.2), which is consistent with the inability of free Mg^{2+}, at 150µM, to stimulate state-4 respiration (see Chapter II, Fig.2.5). However, addition of a much higher concentration of Mg^{2+} (5mM) stimulated the oligomycin-insensitive ATPase activity, at 30°C, in these mitochondria also (Table 3.2); this activity was only 30-50% of that observed with spleen mitochondria, which explains the earlier observation that only a moderate stimulation of state-4 respiration rate was observed, even after the addition of Mg^{2+} to thymus mitochondria at higher concentrations (>3mM; Chapter II, Fig. 2.5).

3.4.2 Effect of oligomycin on ATP-synthase activities from spleen and thymus mitochondria

The matrix ATPases (ATP-synthase or oligomycin-sensitive ATPase) of spleen and thymus mitochondria differed markedly in their sensitivities to oligomycin. In well-coupled spleen mitochondria, the matrix ATPase was inhibited by >90% when oligomycin (2µg/mg) or atractyloside (50µM) were added to the medium separately. On the other hand, the addition of both oligomycin (2µg/mg) and atractyloside (50µM) together completely inhibited the matrix ATPase of intact thymus mitochondria; when oligomycin was added alone it did not inhibit the ATPase activity completely, even at concentrations as high as 10µg/mg (Fig.3.3). If

Fig.3.3 Effect of oligomycin on ATPase activity of thymus mitochondria

ATPase activity was assayed as described in the Experimental section. Oligomycin was present in the incubation medium, at the concentrations given in the Fig. Freshly prepared mitochondrial protein (0.4mg) was added to initiate the reaction (Fig. a) or was preincubated with oligomycin for 15 minutes before use (Fig. b). The values represent an average of 2 experiments.


(a) No preincubation
(b) 15 min preincubation
μmol Pi / mg of protein
3·0
1.5
0
0
10
20
30
Time(min)
CONTROL
2 μg /mg
5 μg / mg
10 μg / mg

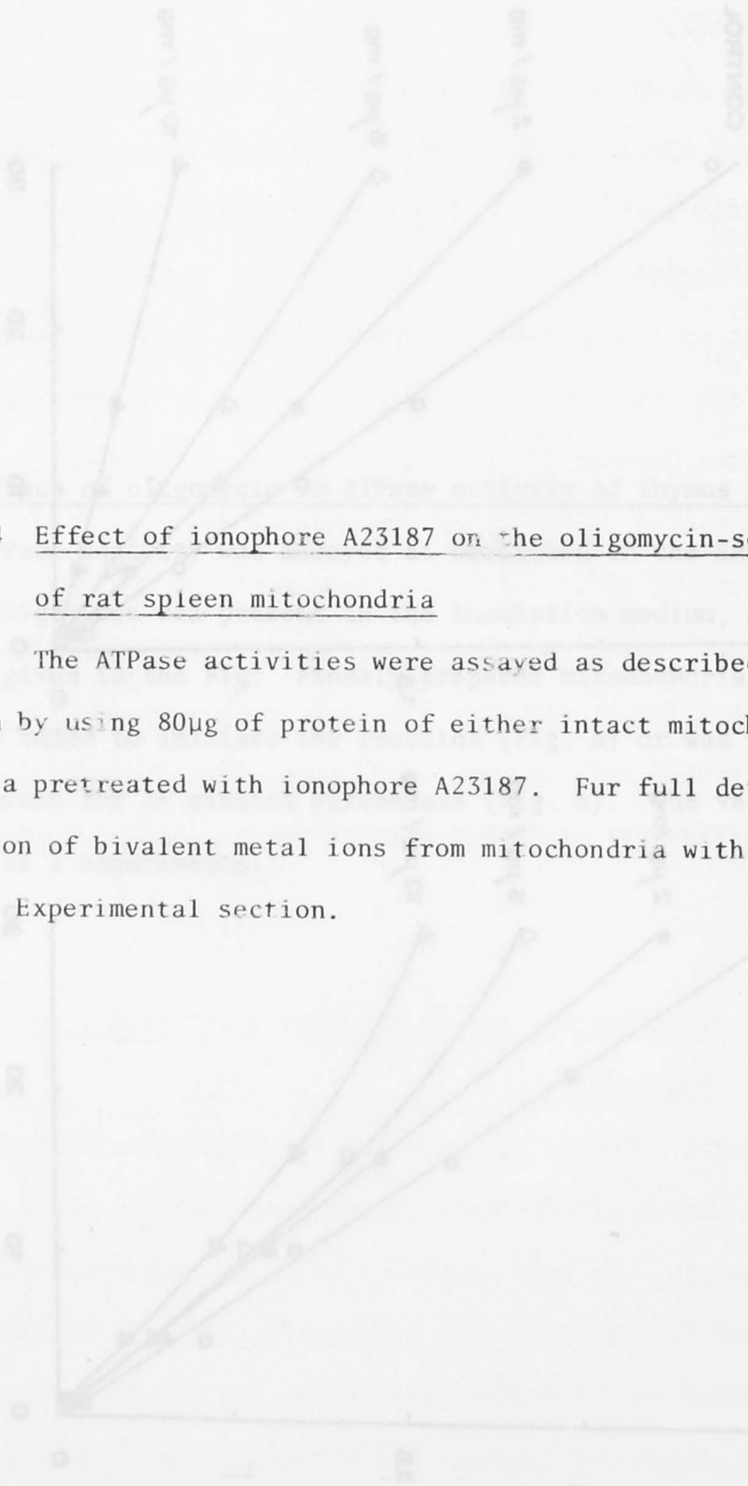
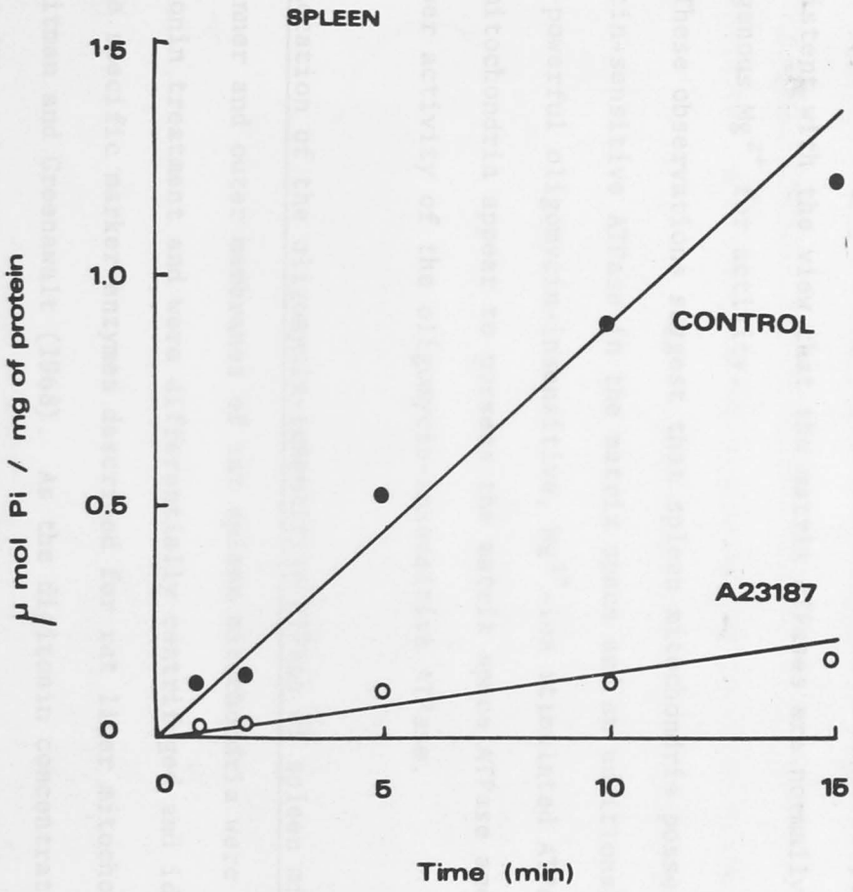
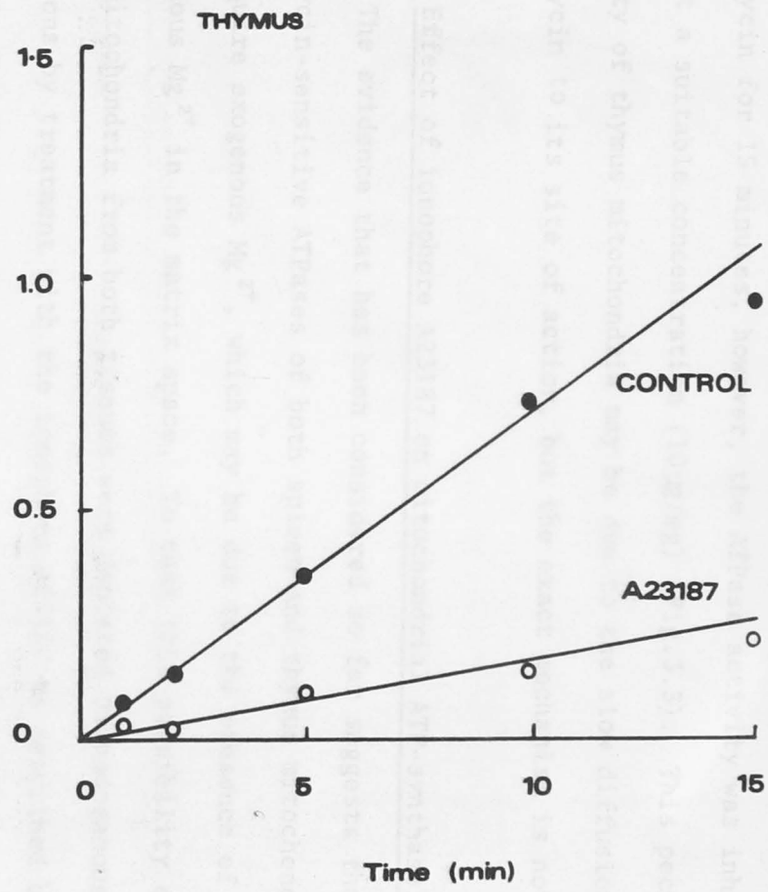


Fig.3.4 Effect of ionophore A23187 on the oligomycin-sensitive ATPase of rat spleen mitochondria

The ATPase activities were assayed as described in the Experimental section by using 80μg of protein of either intact mitochondria or mitochondria pretreated with ionophore A23187. For full details of the depletion of bivalent metal ions from mitochondria with ionophore A23187, see the Experimental section.


SPLEEN
1·5
1.0
0.5
0
μ mol Pi / mg of protein
CONTROL
A23187
0
5
10
15
Time (min)


THYMUS
1·5
1.0
0.5
0
CONTROL
A23187
0
5
10
15
Time (min)

thymus mitochondria were preincubated with different concentrations of oligomycin for 15 minutes, however, the ATPase activity was inhibited >82% at a suitable concentration (10µg/mg) (Fig.3.3). This peculiar property of thymus mitochondria may be due to the slow diffusion of oligomycin to its site of action, but the exact mechanism is not known.

3.4.3 Effect of ionophore A23187 on mitochondrial ATP-synthase activities

The evidence that has been considered so far suggests that the oligomycin-sensitive ATPases of both spleen and thymus mitochondria do not require exogenous Mg^{2+}, which may be due to the presence of adequate endogenous Mg^{2+} in the matrix space. To test this possibility experimentally, mitochondria from both tissues were depleted of endogenous bivalent metal ions by treatment with the ionophore A23187 as described by Reed and Lardy (1972) and their ability to hydrolyze [γ^{32}P]-ATP was tested. Fig.3.4 shows clearly that both spleen and thymus mitochondria lost their Mg^{2+}-independent ATPase activities after treatment with A23187, which is consistent with the view that the matrix ATPases are normally dependent on endogenous Mg^{2+} for activity.

These observations suggest that spleen mitochondria possess an oligomycin-sensitive ATPase in the matrix space and an additional, equally-powerful oligomycin-insensitive, Mg^{2+}-ion stimulated ATPase. Thymus mitochondria appear to possess the matrix space ATPase and a very much lower activity of the oligomycin-insensitive ATPase.

3.4.4 Location of the oligomycin-insensitive ATPase of spleen mitochondria

Inner and outer membranes of rat spleen mitochondria were separated by digitonin treatment and were differentially centrifuged and identified using the specific marker enzymes described for rat liver mitochondria by Schnaitman and Greenawalt (1968). As the digitonin concentration was increased (Fig.3.5a) the activity of monoamine oxidase, the specific

Fig.3.5 Differential fractionation of rat spleen mitochondria with digitonin

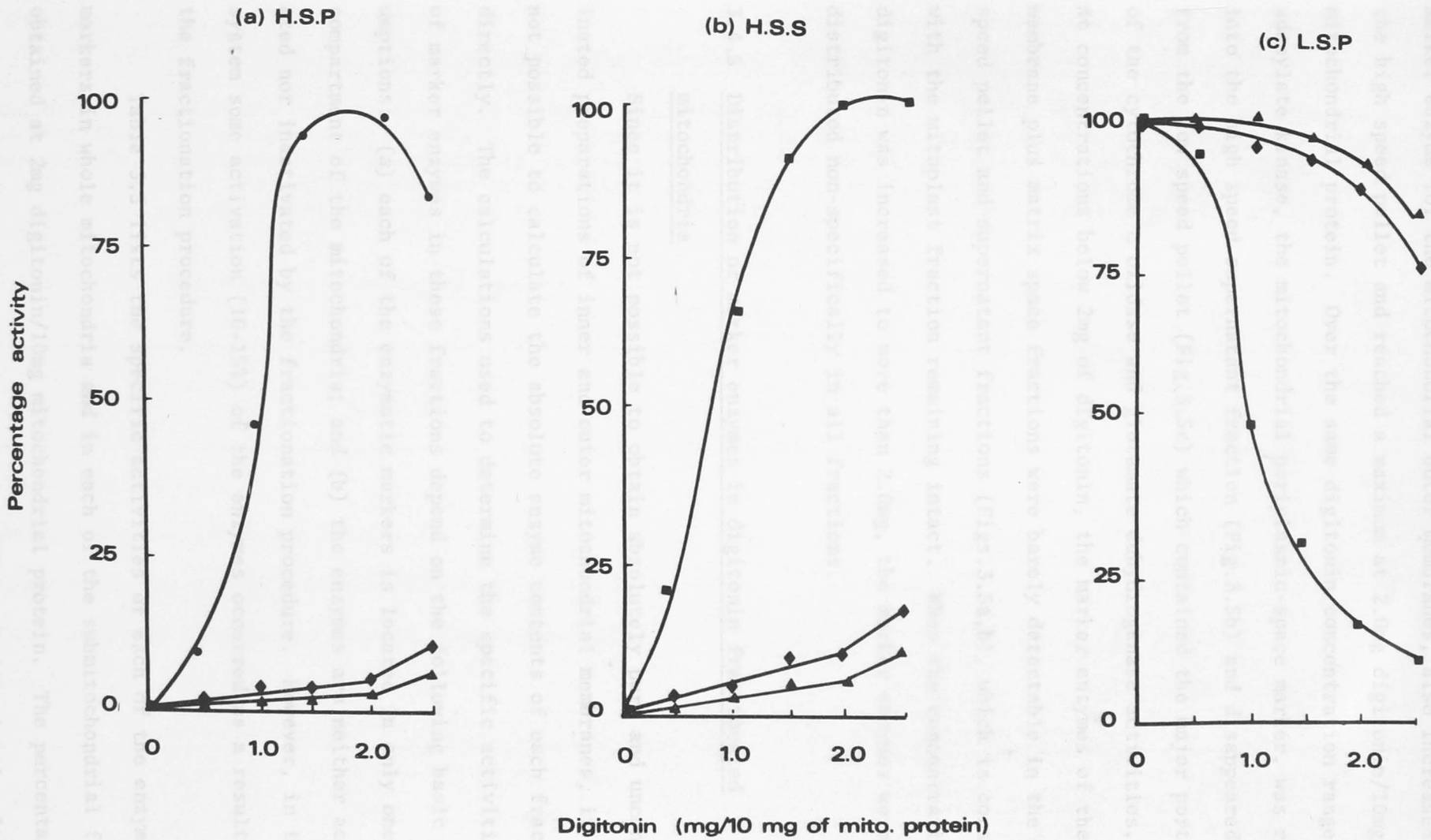
Spleen mitochondria were treated with different concentrations of digitonin (0.5-2.5mg/10mg of protein), and various mitochondrial fractions [outer membrane fraction, H.S.P. (Fig. a), intermembrane soluble fraction, H.S.S. (Fig. b) and inner membrane plus matrix fraction, L.S.P. (Fig. c)] were prepared by differential centrifugation as described in the Experimental section. Specific marker enzymes of different fractions (● , monoamine oxidase; ▲ , cytochrome oxidase; ♦ , glutamate dehydrogenase; ■ , adenylate kinase) were assayed as described in the text.

H.S.P. - High Speed Pellet

H.S.S. - High Speed Supernatant

L.S.P. - Low Speed Pellet

The results represent an average of two different duplicate experiments.


(a) H.S.P
(b) H.S.S
(c) L.S.P
Percentage activity
100
75
50
25
0
0
1.0
2.0
Digitonin (mg/10 mg of mito. protein)

marker enzyme for the mitochondrial outer membranes, also increased in the high speed pellet and reached a maximum at 2.0mg digitonin/10mg mitochondrial protein. Over the same digitonin concentration range, adenylate kinase, the mitochondrial periplasmic-space marker, was released into the high speed supernatant fraction (Fig.3.5b) and disappeared from the low speed pellet (Fig.3.5c) which contained the major portion of the cytochrome c oxidase and glutamate dehydrogenase activities. At concentrations below 2mg of digitonin, the marker enzymes of the inner membrane plus matrix space fractions were barely detectable in the high speed pellet and supernatant fractions (Figs.3.5a,b), which is consistent with the mitoplast fraction remaining intact. When the concentration of digitonin was increased to more than 2.0mg, the marker enzymes were distributed non-specifically in all fractions.

3.4.5 Distribution of marker enzymes in digitonin fractionated mitochondria

Since it is not possible to obtain absolutely pure and uncontaminated preparations of inner and outer mitochondrial membranes, it is not possible to calculate the absolute enzyme contents of each fraction directly. The calculations used to determine the specific activities of marker enzymes in these fractions depend on the following basic assumptions: (a) each of the enzymatic markers is located in only one compartment of the mitochondria; and (b) the enzymes are neither activated nor inactivated by the fractionation procedure. However, in this system some activation (10-15%) of the enzymes occurred as a result of the fractionation procedure.

Table 3.3 lists the specific activities of each of the enzymatic markers in whole mitochondria and in each of the submitochondrial fractions obtained at 2mg digitonin/10mg mitochondrial protein. The percentage of the total mitochondrial protein represented by each fraction is also

Table 3.3

Distribution of specific marker enzymes in digitonin-treated rat spleen mitochondria

Rat spleen mitochondria were treated with 2.0mg of digitonin/10mg of protein and the various fractions were prepared as described in the Experimental section. Mg^{2+}-stimulated ATPase activity was measured in each fraction in the presence of 10µg of oligomycin/mg of protein (3.3C); oligomycin was omitted in the assay for oligomycin-sensitive ATPase (3.3b). The other enzymes were assayed as described in the text. Specific activities of all enzymes are expressed in n mol/min per mg of protein. The numbers of experiments are given in parentheses and the results are mean activities ± S.E.M.

Table (3.3a) Distribution of membrane proteins

FRACTION	PROTEIN (Percentage)
Whole mitochondria	(5) 100
Outer membranes (High Speed Pellet)	7.2 ± 1.7
Inner membranes + Matrix (Low Speed Pellet)	81.2 ± 6.9
Inter-membrane soluble space (High Speed supernatant)	13.9 ± 2.3
Recovery	102.2 ± 3.6

Table 3.3b

Distribution of enzymes of matrix plus inner-membrane

Details are given in the Legends to Table 3.3

FRACTION	GLUTAMATE DEHYDROGENASE		CYTOCHROME-C OXIDASE		ATPase (OLIGOMYCIN-SENSITIVE)	
	(2)		(2)		(3)	
	Specific activity	Total activity	Specific activity	Total activity	Specific activity	Total activity
Whole mitochondria	421	100	1071	100	108 ±11.2	100
Outer membranes (high speed pellet)	1.1	0.2	86	5.7	14.2±1	9.4
Inner membranes + matrix (low speed pellet)	592	114	1476	89.5	147.2±7	110.5
Inter-membrane soluble space (high speed supernatant	4.7	1.5	129	15.6	0	0
Recovery	-	115.7	-	110.8	-	119.9

Table 3.3c

Distribution of enzymes of outer membrane

Details are given in the Legends to Table 3.3

FRACTION	MONOAMINE OXIDASE		Mg^{2+}-ATPase (OLIGOMYCIN-INSENSITIVE)	
	Specific activity	(3) Total activity	Specific activity	(8) Total activity
Whole mitochondria	12.1± 2.4	100	178± 6.7	100
Outer membranes (high speed pellet)	147.0±17.1	87.5	2318±51.6	89.3
Inner membranes + matrix (low speed pellet)	2.9± 0.4	19.4	16.9±3.2	8.1
Inter-membrane soluble space (high speed supernatant)	-	-	0	0
Recovery	-	106.9	-	97.4

Table 3.3d

Distribution of enzymes of soluble inter-membrane space

Details are given in the Legends to Table 3.3

FRACTION	ADENYLATE KINASE		CREATINE-KINASE		INORGANIC PYROPHOSPHATASE	
	Specific activity (3)	Total activity	Specitic activity (3)	Total activity	Specific activity (2)	Total activity
Whole mitochondria	287 ±11.1	100	76.5 ± 6.1	100	317.6	100
Outer membranes (high speed pellet)	14.2± 3	5.2	1.3 ± 0.2	1.7	10.2	2.3
Inner membranes + matrix (low speed pellet)	46 ± 3	13.7	2.98± 0.5	3.9	11.3	2.9
Inter-membrane soluble space (high speed supernatant)	2032 ±95	103.8	561.9 ±37	102.7	2202.6	96.4
Recovery	-	117.5	-	108.3	-	101.6

given in Table (3.3a), and was calculated with the following formula:

$$\text{Percentage of total mitochondrial protein present in a fraction} = \left\{ \frac{\text{Sp. activity of marker enzyme in whole mitochondria}}{\text{Sp. activity of marker enzyme in the particular fraction.}} \right\} \times 100$$

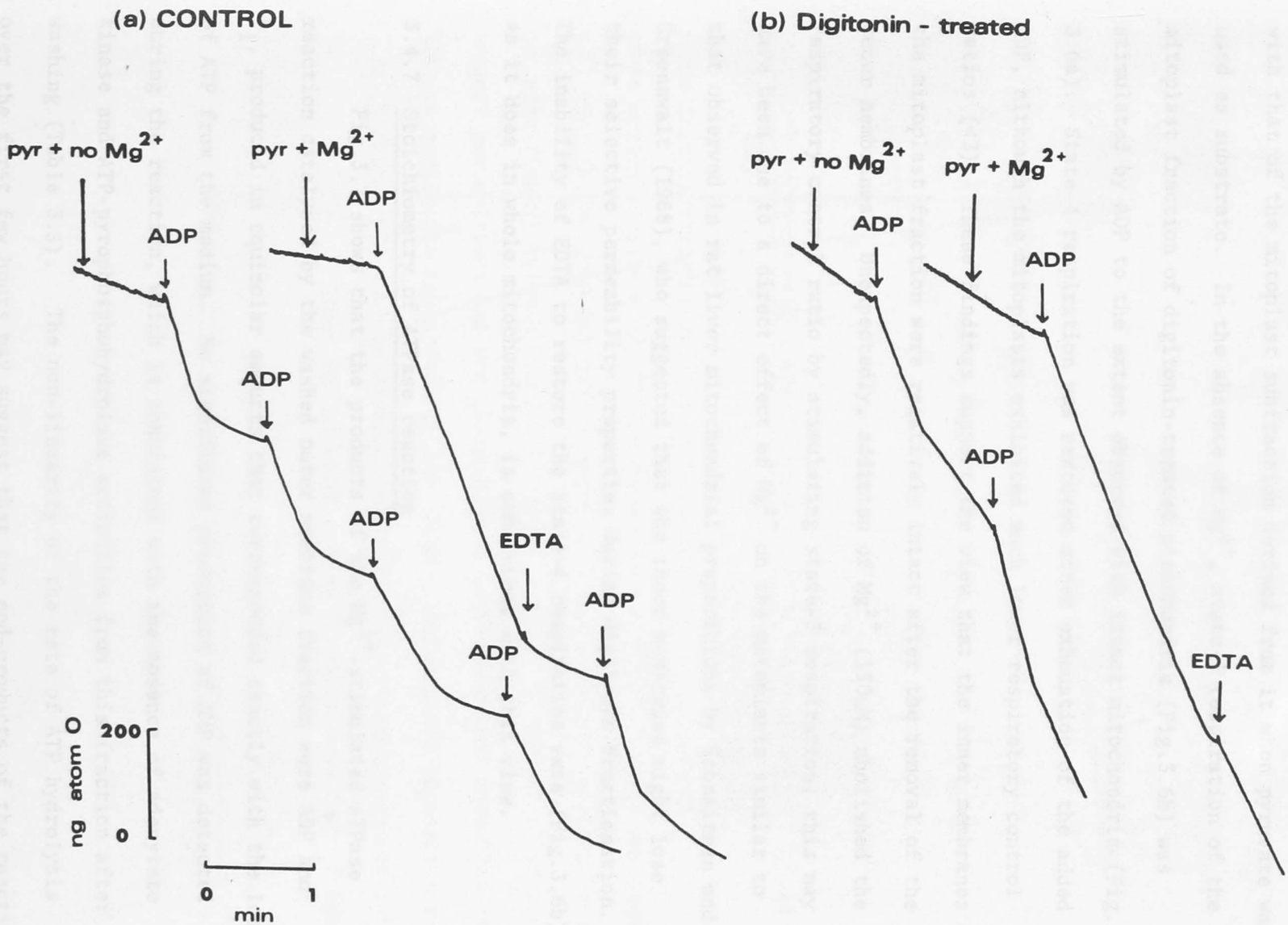
The sum of the percentages of the protein present in each fraction calculated in this fashion is almost equal to 100%, which supports the general validity of the assumptions used.

More than 80% of the total mitochondrial protein remained in the low speed pellet, which also contained elevated specific activities of the marker enzymes of the inner membrane plus matrix space fractions (Table 3.3b). Only 7-8% of the total mitochondrial protein was recovered in the high speed pellet; in this fraction a selective 9 to 12-fold enhancement of the specific activities of monoamine oxidase and the oligomycin-insensitive ATPase indicated that both of these activities copurified with the outer membrane subfraction (Table 3.3c). Approximately 11-13% of the total mitochondrial protein was released into the inter-membrane soluble space fraction which contained inorganic pyrophosphatase, adenylate kinase and creatine kinase of greatly enhanced specific activity (7-fold) (Table 3.3d).

Of the various fractions obtained by digitonin treatment, approximately 90% of the total oligomycin-insensitive ATPase activity was recovered with the outer membranes (Table 3.3c). The small amount of activity (8%) still associated with the inner membranes is a result, possibly, of incomplete separation of the two membranes (Table 3.3b). Taken together, these results indicate that the oligomycin-insensitive, Mg^{2+}-stimulated ATPase of spleen mitochondria is localized predominantly in the outer membrane fraction whilst (as expected) the oligomycin-sensitive ATPase is located almost exclusively in the inner membrane plus matrix space.

Fig.3.6 Respiration of digitonin-treated mitochondria

Intact mitochondria from spleen (1.2mg; Fig.a) or the digitonin-fractionated mitochondria (1.5mg; Fig.b) were used to measure respiration which was monitored as described in the Experimental section. Pyruvate (3.0mM) plus malate (0.5mM), $MgCl_2$ (0.9mM), EDTA (600µM) and ADP (200µM) were added to the medium as indicated.


(a) CONTROL
pyr + no Mg2+
pyr + Mg2+
ADP
ADP
ADP
ADP
EDTA
ADP
ADP
ng atom O
200
0
0
1
min
(b) Digitonin - treated
pyr + no Mg2+
pyr + Mg2+
ADP
ADP
ADP
EDTA

3.4.6 Respiration of digitonin-fractionated spleen mitochondria

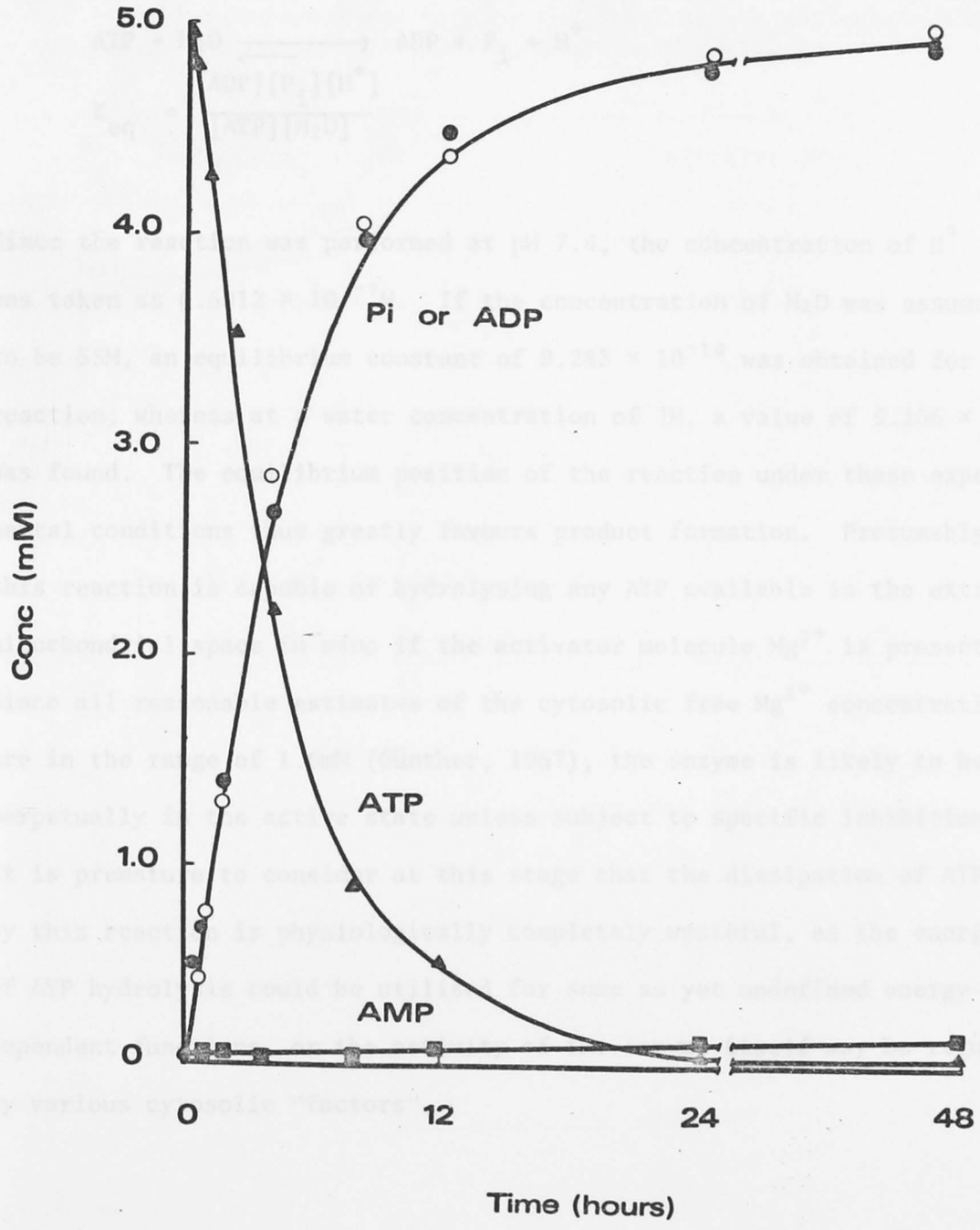
Fig. 3.6 compares the respiration of intact spleen mitochondria with that of the mitoplast subfraction derived from it when pyruvate was used as substrate. In the absence of Mg^{2+}, state-3 respiration of the mitoplast fraction of digitonin-treated mitochondria (Fig.3.6b) was stimulated by ADP to the extent observed with intact mitochondria (Fig. 3.6a). State-4 respiration was restored after exhaustion of the added ADP, although the mitoplasts exhibited much lower respiratory control ratios (<2). These findings support the view that the inner membranes of the mitoplast fraction were relatively intact after the removal of the outer membranes. Unexpectedly, addition of Mg^{2+} (150μM) abolished the respiratory control ratio by stimulating state-3 respiration; this may have been due to a direct effect of Mg^{2+} on the mitoplasts similar to that observed in rat liver mitochondrial preparations by Schnaitman and Greenawalt (1968), who suggested that the inner membranes might lose their selective permeability properties during digitonin fractionation. The inability of EDTA to restore the state-4 respiration rate (Fig.3.6b), as it does in whole mitochondria, is consistent with this view.

3.4.7 Stoichiometry of ATPase reaction

Fig.3.7 shows that the products of the Mg^{2+}-stimulated ATPase reaction catalysed by the washed outer membrane fraction were ADP and P_i, produced in equimolar amounts that corresponded exactly with the loss of ATP from the medium. No significant production of AMP was detected during the reaction, which is consistent with the absence of adenylate kinase and ATP-pyrophosphohydrolase activities from this fraction after washing (Table 3.5). The non-linearity of the rate of ATP hydrolysis over the first few hours may suggest that the end-products of the reaction, such as ADP or P_i exert an inhibitory effect on the enzyme reaction.

Fig.3.7 Stoicheiometry of the outer membrane ATPase reaction from rat spleen mitochondria

Hydrolysis of unlabelled ATP by the washed mitochondrial outer membrane (200µg) ATPase was performed at 37°C in a sterile medium containing tetracycline (2mg/ml) as well as Hepes buffer (20.0mM, pH7.4), ATP (5.0mM) and $MgCl_2$ (8.0mM) in a final volume of 5.0ml. Samples (200µl) were withdrawn at different time-intervals, as indicated, and the reaction was stopped with ice-cold $HClO_4$ (20µl, 1.0M). All glassware was autoclaved at 120°C for 30 minutes before use. Adenine nucleotides (▲, ATP; ○, ADP; ■, AMP) and P_i (●) were analysed as described in the text.


5.0
4.0
3.0
2.0
1.0
0
Conc (mM)
Pi or ADP
ATP
AMP
0
12
24
48
Time (hours)

The reaction was allowed to go to completion (in a sterile medium to prevent the non-specific hydrolysis of ATP by contaminating bacteria) in order to calculate its equilibrium position. The following equation was used:

$$ATP + H_2O \rightleftharpoons ADP + P_i + H^+$$

$$K_{eq} = \frac{[ADP][P_i][H^+]}{[ATP][H_2O]}$$

Since the reaction was performed at pH 7.4, the concentration of H^+ was taken as 0.5012×10^{-7}M. If the concentration of H_2O was assumed to be 55M, an equilibrium constant of 9.283×10^{-10} was obtained for this reaction; whereas at a water concentration of 1M, a value of 5.106×10^{-8} was found. The equilibrium position of the reaction under these experimental conditions thus greatly favours product formation. Presumably this reaction is capable of hydrolysing any ATP available in the extramitochondrial space *in vivo* if the activator molecule Mg^{2+} is present. Since all reasonable estimates of the cytosolic free Mg^{2+} concentration are in the range of 1.6mM (Günther, 1967), the enzyme is likely to be perpetually in the active state unless subject to specific inhibition. It is premature to consider at this stage that the dissipation of ATP by this reaction is physiologically completely wasteful, as the energy of ATP hydrolysis could be utilised for some as yet undefined energy-dependent functions, or the activity of the enzyme itself may be regulated by various cytosolic "factors".

Table 3.4 .

Effect of various inhibitors on the outer membrane ATPase activity of spleen mitochondria

ATPase activity was assayed as described in the Experimental section by using intact spleen mitochondria (100µg of protein), except that atractyloside (50µM) and $MgCl_2$ (5mM) were present in the reactin mixture in all experiments. Inhibitors were added to give the final concentrations indicated in the Table. Values are given as an average of duplicate experiments.

ADDITION		ACTIVITY (%)
None		100
Oligomycin (µg/mg protein)	5.0	98.1
	10.0	92.7
Mersalyl (mM)	0.005	93.7
	0.010	87.3
Ouabain (mM)	0.01	96.7
	0.05	92.1
	0.10	91.7

Table 3.5

Effect of washing the outer membrane preparation from rat spleen mitochondria on the disappearance of contaminating enzyme activities

Mitochondrial outer membranes were prepared as described in the Experimental section and were washed between spins with Hepes buffer (20.0mM; pH 7.4) before sedimentation at 100,000 × g for 60 minutes. The enzymes listed in the Table were assayed as described in the text. All values are expressed as a percentage of the activity measured with intact mitochondria (given as 100%); specific activity is expressed as n mol/min/mg protein. The number of observations are given in parentheses and the results are expressed as mean activities ± S.E.M.

PREPARATION ASSAYED	ADENYLATE KINASE	CREATINE KINASE	INORGANIC PYROPHOSPHATASE	ATP-PYROPHOSPHO-HYDROLASE	5'-NUCLEOTIDASE
	(3)	(3)	(2)	(2)	(2)
Intact mitochondria					
Specific activity	287 ± 11.1	76.5 ± 6.1	317.6	0	0
Percentage activity	100%	100%	100%	-	-
Outer membranes					
i Unwashed	5.2%	1.7%	2.3%	0	0
ii After 3 washes	0%	0%	0.04%	0	0

3.4.8 Examination of the outer membrane fraction for contamination with other ATPase activities

Outer membrane preparations were tested for possible plasma membrane contamination, using Na^+, K^+-ATPase as the marker enzyme, by assaying the Mg^{2+}-stimulated ATPase activity in the presence of ouabain (0.01 - 0.1 mM). Ouabain had no significant effect on the Mg^{2+}-stimulated activity when tested over this concentration range, (Table 3.4) which is consistent with the absence of plasma membrane contamination. This conclusion was strengthened by the total absence of 5'-nucleotidase activity from intact spleen mitochondria and from all of the mitochondrial sub-fractions (Table 3.5). Similarly, mersalyl, which is a specific potent inhibitor of sarcoplasmic reticulum ATPase, had no measurable effect on the Mg^{2+}-stimulated ATPase activity (Table 3.4). As seen earlier in this chapter, the insensitiveness of this reaction to the addition of oligomycin indicates that the inner membranes were virtually absent from the outer membrane preparations.

Of other possible contaminating activities, only 2% of the total creatine kinase activity, measured in the direction of ATP synthesis from ADP and creatine phosphate, was present in the unwashed outer membrane fraction. A similar proportion of the inorganic pyrophosphatase activity, which also copurified with adenylate kinase and creatine kinase from the periplasmic space, was found in this sub-fraction. Table 3.5 shows that all of these contaminating activities disappeared from the outer membrane preparations after three washes.

3.4.9 Effect of "ageing" on spleen mitochondrial ATPase activity

Rat spleen mitochondria were isolated, as described in the text, as fast as possible: the time lapse was 20 minutes from the time of removal of the spleens to the completion of the isolation procedure.

Table 3.6 .

Effect of ageing of rat spleen mitochondria on the activity of Mg^{2+}-stimulated ATPase

Mitochondria from rat spleen were isolated essentially as described in the Methods Section, but with some modifications. Spleens were removed from the animals as quickly as possible and were placed in ice-cold isolation medium, homogenized immediately and differentially centrifuged to obtain mitochondria. The mitochondrial pellet was washed only once with wash medium and was used for ATPase assay as described in the text, except that ouabain (0.1mM) was present in the incubation medium in addition to oligomycin (10µg/mg protein) and atractyloside (50µM). The values represent an average of two experiments.

TIME (hrs)	ATPase ACTIVITY (n mols/min/mg protein)
0	171.7
½	176.4
1	168.2
2	170.6
4	178.9
5	174.3

Table 3.7

Tissue distribution of the outer membrane ATPase activity

Mitochondria from different rat tissues were isolated as described in the text. The mitochondrial respiration was measured in an incubation medium containing either (i) no added bivalent metal ions or (ii) $MgCl_2$ (0.9mM) or (iii) $CaCl_2$ (0.6mM). The ATPase activity was assayed in an incubation medium containing [γ-^{32}P]-ATP (0.15mM at a specific activity of 1.5×10^6 d.p.m./μ mol), atractyloside (50μM) and either $MgCl_2$ (0.9mM) or $CaCl_2$ (0.6mM) in a final volume of 3.0ml.

TISSUE (MITOCHONDRIA)	STATE-4 RESPIRATION (ng atom O/min/mg)			ATPase ACTIVITY (nmol Pi/min/mg)	
	No bivalent metal ions	$MgCl_2$ (0.9mM)	$CaCl_2$ (0.6mM)	$MgCl_2$ (0.9mM)	$CaCl_2$ (0.6mM)
liver	21	28	98	12	4
spleen	25	108	151	150	65
thymus	32	41	115	14	7
heart	30	142	158	760	442
brain	28	104	114	17	14
kidney-cortex	31	89	97	71	47
lung	23	59	120	12	19
hind-leg muscle	29	31	117	12	27
diaphragm	34	37	106	9	29

Using these mitochondria, the Mg^{2+}-stimulated ATPase activity was measured immediately and this was considered to be the zero time activity. The mitochondria were left on ice at 0°C and small samples were removed at the desired time intervals and assayed for Mg^{2+}-stimulated ATPase. The results in Table 3.6 indicate that the observed ATPase activity was not associated with "ageing" of mitochondria, as the activity remained unchanged throughout the 5 hour period after the isolation of the mitochondria.

3.4.10 Tissue distribution

Well-coupled mitochondria from various tissues were prepared by standard methods and their respiration studied in the presence of succinate in an incubation medium free from added bivalent metal ions; the effect of adding Mg^{2+} or Ca^{2+} was tested once state-4 respiration had been established. Table 3.7 shows that Mg^{2+} (0.9mM) stimulated state-4 respiration in only kidney-cortex, brain, heart and spleen mitochondria, whilst Ca^{2+} (0.6mM) invariably stimulated the rate in all mitochondrial preparations due to its uptake at the expense of ATP hydrolysis. In subsequent experiments, where the ATPase activity was assayed by following the specific release of $^{32}P_i$ from [γ^{32}P]-ATP in the presence of atractyloside, the results show clearly that only spleen, kidney-cortex and heart mitochondria possessed this distinctive ATPase activity (see Table 3.7). The highest specific activity was observed with heart mitochondria, which exceeded that of spleen by at least six-fold. Brain mitochondria did not release $^{32}P_i$ under these conditions, so in this case the Mg^{2+}-stimulated state-4 rate observed is more likely to be due to the stimulation of a phosphotransferase activity like creatine kinase (Sugano and Nagai, 1971). Under these assay conditions (Mg^{2+}, 0.9mM; temperature, 22°C) thymus mitochondria showed no significant activity (see Table 3.7).

3.5 DISCUSSION

3.5.1 Purity of the isolated rat spleen mitochondria

Isolated mitochondria are usually contaminated, to some extent, with other cytoplasmic organelles, such as microsomes, lysosomes, sarcoplasmic or endoplasmic reticulum, or with plasma membranes. In the present study, care was taken to minimize such contamination in the following ways: (1) the 650 × g nuclear pellet was not resuspended to extract the residual mitochondria, in order to reduce nuclear contamination; (2) addition of bovine serum albumin to the isolation medium which is beneficial for the stability of the membranes during homogenization (Munn, 1976), also increases the co-sedimentation of other cytoplasmic organelles (e.g., microsomes, lysosomes) with mitochondria (Kun, 1976); based on these findings, bovine serum albumin was excluded from the wash medium but not from the isolation medium; (3) the mitochondrial pellet was dispersed by hand with a losse-fitting homogenizer and washed at least three times to minimize the adsorption of microsomal and plasma membrane contaminants. The measurement of low activities (<10%) of marker enzymes for plasma membranes (5'-nucleotidase - see Table 3.5), microsomes and sarcoplasmic reticulum (mersalyl-sensitive Ca^{2+}, Mg^{2+}-ATPase - see Table 3.4; MacLennan, 1970) indicates that the isolated spleen mitochondria were relatively free of other sub-cellular fractions.

3.5.2 Homogenity of the membrane preparations from digitonon-treated rat spleen mitochondria

The extensively folded inner membranes (cristae) represent 75% of the total protein content of mitochondria, whereas the surrounding thin outer membranes comprise only 6% of the protein (see Table 3.3a). Isolation of a homogenous preparation of the outer membranes without contamination from the more abundant inner membranes has often proved to be difficult. In principle, each membrane can be identified and

isolated selectively by following the enrichment of its specific marker enzymes. The results of the present study indicate that the outer membrane fraction contained more than 85% of the monoamine oxidase activity and less than 10% of the enzymes of the other mitochondrial fractions (see Table 3.3). The inner membrane fraction was enriched in its specific marker enzymes (e.g., glutamate dehydrogenase, oligomycin-sensitive ATPase and cytochrome c oxidase). Although only 1-2% of the glutamate dehydrogenase and oligomycin-sensitive ATPase activities were recovered in the high speed supernatant (intermembrane space) fraction, more than 15% of the cytochrome c oxidase activity was detected in this fraction. Cytochrome c oxidase is only loosely bound to the inner membranes of rat lymphoid tissue mitochondria (Scaife, 1966), and is evidently detached from the membranes to some extent during fractionation. If the inner membranes had been broken, the membrane-bound cytochrome c oxidase activity would have been recovered with the outer membranes rather than with the high speed supernatant. Furthermore, the inner membrane plus matrix-space fraction gave respiratory control ratios of about 2.0, which is consistent with the intactness of the inner membrane.

3.5.3 Nature of the ADP-regenerating reaction in Mg^{2+}-treated spleen mitochondria

The apparent loss of phosphorylation efficiency (ADP/O ratio) and the fall in the acceptor control ratio observed when Mg^{2+} is added to rat spleen mitochondria is similar to that reported for heart sarcosomes (Packer, 1957, 1958; Chao and Davis, 1972) and rat kidney-cortex mitochondria (Gmaj *et al.*, 1974). The observation in rat kidney-cortex mitochondria has been attributed to the stimulation of a Mg^{2+}-dependent ATPase from the outer membrane fraction (Gmaj *et al.*, 1974). In our hands, preliminary studies with spleen mitochondria have indicated a similar type of activity stimulated by Mg^{2+}, in the atractyloside-insensitive space (see Fig.3.1).

In the case of heart mitochondria it has been suggested that the external ATPase, which is active only in the presence of Mg^{2+}, may be a contaminant that is identical with the ATPase-coupling factor of oxidative phosphorylation (Chao and Davis, 1972). This conclusion was reached on the basis of the sensitivity of the reaction to inhibition by oligomycin, but no attempts were made to study its location in the mitochondrial subfractions. If such an active, Mg^{2+}-stimulated enzyme were present due to mitochondrial damage and subsequent release of ATP-synthase activity, then the efficiency of oxidative phosphorylation and respiratory control ratios should be very low. In contrast, high respiratory control ratios (about 8-12) were reported (Chao and Davis, 1972). Secondly, under these conditions most of the matrix space enzymes should have leaked out during the washing procedure and consequently the mitochondria should have had a poor capacity to oxidize tricarboxylic acid cycle intermediates. High rates of oxidation of glutamate have in fact been reported. If, on the other hand, only slight damage to the inner membranes has occurred, this cannot account for the high rates of Mg^{2+}-stimulated ATPase observed both in the present study (see Table 3.7) and in the experiments of Chao and Davis (1972).

The contribution of ATPase or ATPase-like activities from other cellular sub-fractions such as the sarcoplasmic reticulum, actomyosin from contractile fibres, creatine kinase or glucose-hexokinase of microsomes have been ruled out as possible causal factors in the experiments of Chao and Davis (1972). The available evidence does not rule out the possibility that the Mg^{2+}-stimulated ATPase activity of heart mitochondria may be associated with the outer membranes, although there is no direct evidence to support this conclusion.

In the present study, the following factors are considered to support the view that the Mg^{2+}-stimulated uncoupling effect in rat spleen mitochondria is due to the stimulation of an outer membrane ATPase. Mito-

chondrial damage was excluded on the following grounds: (a) high respiratory control and ADP/O ratios were obtained with the same mitochondrial preparations in the absence of added Mg^{2+} (Fig.3.6); (b) the Mg^{2+}-independent ATPase activity was suppressed completely by atractyloside (Fig.3.1); (c) the atractyloside-insensitive ATPase activity, which required added Mg^{2+} for activation, was quite insensitive to oligomycin (Table 3.1) which argues against it being a contaminating ATPase activity released from the mitochondrial matrix; and (d) the possibility that the addition of Mg^{2+} itself might cause an irreversible loss of mitochondrial integrity, and a concomitant stimulation of state-4 respiration, was ruled out by the restoration of both normal state-4 respiration and the cyclic response of the mitochondria to the addition of ADP on subsequent addition of excess EDTA (Fig. 3.5). In addition, the differential fractionation of spleen mitochondria with digitonin (Table 3.3) has shown clearly that the outer membrane fraction has more than 90% of the oligomycin-insensitive ATPase activity associated with it, which argues for a distinctive activity, not associated with mitochondrial damage, that is typical of spleen mitochondria. Its presence and level of activity is not associated with "ageing" of the mitochondria, as there was neither an increase nor decrease in the Mg^{2+}-stimulated ATPase activity measured in the presence of oligomycin (10μg/mg protein), atractyloside (50μM) and ouabain (0.1mM) over a five hour period after isolation of the mitochondria (Table 3.6).

A number of other possible contaminating activities have been considered, and excluded, as the basis for a Mg^{2+}-stimulated ATPase reaction situated predominantly in the outer membrane fraction. (The small percentage of this activity found in association with the inner membranes is considered to be due to incomplete separation of the two membranes.) The combined activities of ATP-pyrophosphohydrolase (EC 3.6.1.8.) and adenylate kinase (EC 2.7.4.3.), for example, could

result in a "pseudo-ATPase" activity and produce ADP by the following reaction sequence:

$$ATP + H_2O \xrightarrow{\text{ATP-pyrophosphohydrolase}} AMP + PPi \qquad (i)$$

$$AMP + ATP \xrightarrow{\text{adenylate kinase}} 2\ ADP \qquad (ii)$$

Two findings argue against this being the reaction mechanism: (i) the fact that stoicheiometric amounts of ADP and P_i were produced as the only end products of the reaction catalysed by the outer membrane fraction (Fig.3.7); and (ii) the virtual absence of ATP-pyrophosphohydrolase, adenylate kinase and pyrophosphatase activities from the outer membrane fraction after three washes (Table 3.5). Stimulation of creatine kinase activity by added magnesium could also result in a continuous regeneration of ADP from ATP with an apparent uncoupling effect, as has been reported for rat cerebral cortex mitochondria (Sugano and Nagai, 1971); but this activity is completely absent from the washed outer membrane fraction of spleen mitochondria. The outer membrane fraction, in fact, was virtually free of all matrix, inner membrane and inter-membrane space marker enzymes and was selectively enriched in monoamine-oxidase as well as the oligomycin-insensitive ATPase activity (see Table 3.3). The Na^+, K^+-dependent ATPase from the plasma membrane, which may have partially sedimented with the microsomal fraction, was not a contaminant of the outer membrane fraction. These findings indicate that the oligomycin-insensitive ATPase of rat spleen is a distinctive mitochondrial enzyme present in the outer membrane fraction which is similar to the enzyme reported in rat kidney cortex mitochondria by Gmaj *et al.* (1974).

CHAPTER 4

KINETIC PROPERTIES OF THE ATPase FROM THE OUTER MEMBRANE OF RAT SPLEEN MITOCHONDRIA

CHAPTER 4

KINETIC PROPERTIES OF THE ATPase FROM THE OUTER MEMBRANE OF RAT SPLEEN MITOCHONDRIA

4.1 INTRODUCTION

It is evident from the findings reported in the last chapter that rat spleen mitochondria, in common with those from rat kidney-cortex (Gmaj *et al.*, 1974) and possibly heart have a highly active, but oligomycin-insensitive, Mg^{2+}-stimulated ATPase associated exclusively with the outer membrane fraction (see also Vijayakumar and Weidemann, 1976). This enzyme is absent from the outer membranes of rat liver mitochondria which do, however, contain a bicarbonate-stimulated ATPase activity (Grisolia and Mendelson, 1974) that may be an enzyme of the same class.

The oligomycin-sensitive ATPases and purified F_1-preparations from mammalian heart and liver mitochondria (Cooper and Lehninger, 1957) and the ATPases of chloroplasts (Vambutas and Racker, 1965) and bacteria (Adolfsen and Moudrianakis, 1973) that participate in oxidative phosphorylation share the common property of catalyzing maximum ATP hydrolysis with Mg^{2+} as the sole added metal ion. These ATPases can be distinguished from the transport ATPases by virtue of differences in their metal-ion requirements and in their sensitivities to specific inhibitors. For instance, the sarcoplasmic-reticulum ATPase, which is inhibited by mersalyl (MacLennan, 1970), and the erythrocyte-membrane-bound ATPase, which has been implicated in Ca^{2+} transport (Walter and Hasselbach, 1973; Schartzmann and Rossi, 1971), both have an absolute requirement for Ca^{2+} as well as Mg^{2+} for optimum activity. Similarly, the $Na^+ + K^+$-ATPase of the mammalian plasma membrane requires both Na^+ and K^+ in addition to Mg^{2+} to elicit its maximum activity (Glynn, 1956; Post *et al.*, 1960) and is inhibited specifically by ouabain.

As a first step towards defining the distinctiveness and the possible functional significance of the spleen mitochondrial outer membrane ATPase, the influence of metal ions and inhibitors on the kinetic properties of this enzyme have been studied both *in situ* and in isolated outer membrane vesicles. The close resemblance between this enzyme and an ATPase observed in the outer membranes of rat kidney-cortex mitochondria by Gmaj *et al.* (1974), which is thought to be involved in Ca^{2+} transport, may require a reconsideration of the selective permeability properties of the outer membranes of mitochondria that possess enzymes of this type.

4.2 MATERIALS

$[^{32}p]p_i$, digitonin, oligomycin, atractyloside and $CaCl_2$ were obtained from the sources described in the previous Chapters. Norit-A (activated charcoal), bovine serum albumin (fraction V), mersalyl {0 -[(3-Hydroxymercuri-2 methoxypropyl) - carbamyl]-phenoxyacetic acid} and nitrilotriacetic acid, trisodium salt (NTA) were from Sigma Chemical Co., St. Louis, MO, U.S.A. N, N-Dicyclohexylcarbodiimide was from Calbiochem, Carlingford, N.S.W., Australia. $AgNO_3$ was from BDH Chemicals Ltd, Poole, Dorset, U.K. All other chemicals used were of analytical grade.

4.3 METHODS

4.3.1 Isolation of rat spleen mitochondria

Mitochondria from rat spleen were isolated in iso-osmotic sucrose solution as described in the previous chapters, except that the wash medium was free of bovine serum albumin to avoid interference with the mitochondrial protein estimation.

4.3.2 Preparation of spleen mitochondrial outer membrane

The outer membrane fraction from rat spleen mitochondria was prepared as described in chapter III, by using 2.0mg of digitonin/10mg of mitochondrial protein.

4.3.3 Measurement of protein

Mitochondrial and outer membrane protein concentrations were determined as described in Chapter III.

4.3.4 SYNTHESIS OF [γ-^{32}P]ATP

[γ-^{32}P]ATP was synthesized as described earlier (Chapter III).

4.3.5 Assay of ATPase activity

(a) With intact mitochondria: ATPase activity was assayed at 30°C in Eppendorf centrifuge tubes. The reaction medium contained sucrose (250mM), Hepes (25mM; pH adjusted to 7.4 with KOH), unlabelled ATP (2.0mM), [γ-^{32}P]ATP (1.2×10^5 - 1.5×10^5 d.p.m.) and $MgCl_2$ (5.0mM) in a final volume of 0.5ml. The reaction was started by addition of 80-100µg of mitochondrial protein. (b) With the isolated outer membrane preparation: The reaction medium used was identical with that for (a) except that sucrose was replaced by water. The reaction was started by addition of the mitochondrial outer membrane preparation (40-50µg of protein).

The reaction was stopped at different time intervals (usually every 10 mins unless shown otherwise in the legends) by adding ice-cold $HClO_4$ (100µl; 1.0M), and the tubes were placed on ice. The mixture was centrifuged (2 min at 5000g) in an Eppendorf centrifuge to sediment the precipitated protein. The total radioactivity in a sample (100µl) of clear supernatant was determined. Activated charcoal (20mg) was added to the remaining portion of the reaction mixture and mixed for 20 s with a Vortex-Genie mixer, to adsorb the unchanged labelled ATP. The mixture was centrifuged again (2 min at 5000g) to sediment the charcoal. The radioactivity in an equivalent volume (100µl) of clear supernatant which now contained only [^{32}P]P_i was determined. The labelled P_i liberated was

always corrected for the zero-time blank value ($HClO_4$ was added to the reaction medium prior to the addition of enzyme protein). The ATPase activity was linear for more than 10 min, if the protein concentrations indicated were used. Although the results obtained were quite comparable with those described in chapter III, the present method proved to be less time-consuming and equally reproducible. The ^{32}P radioactivity was determined in a Beckman LS-350 liquid scintillation counter as described before (Chapter III).

4.3.6 Measurement of mitochondrial respiration

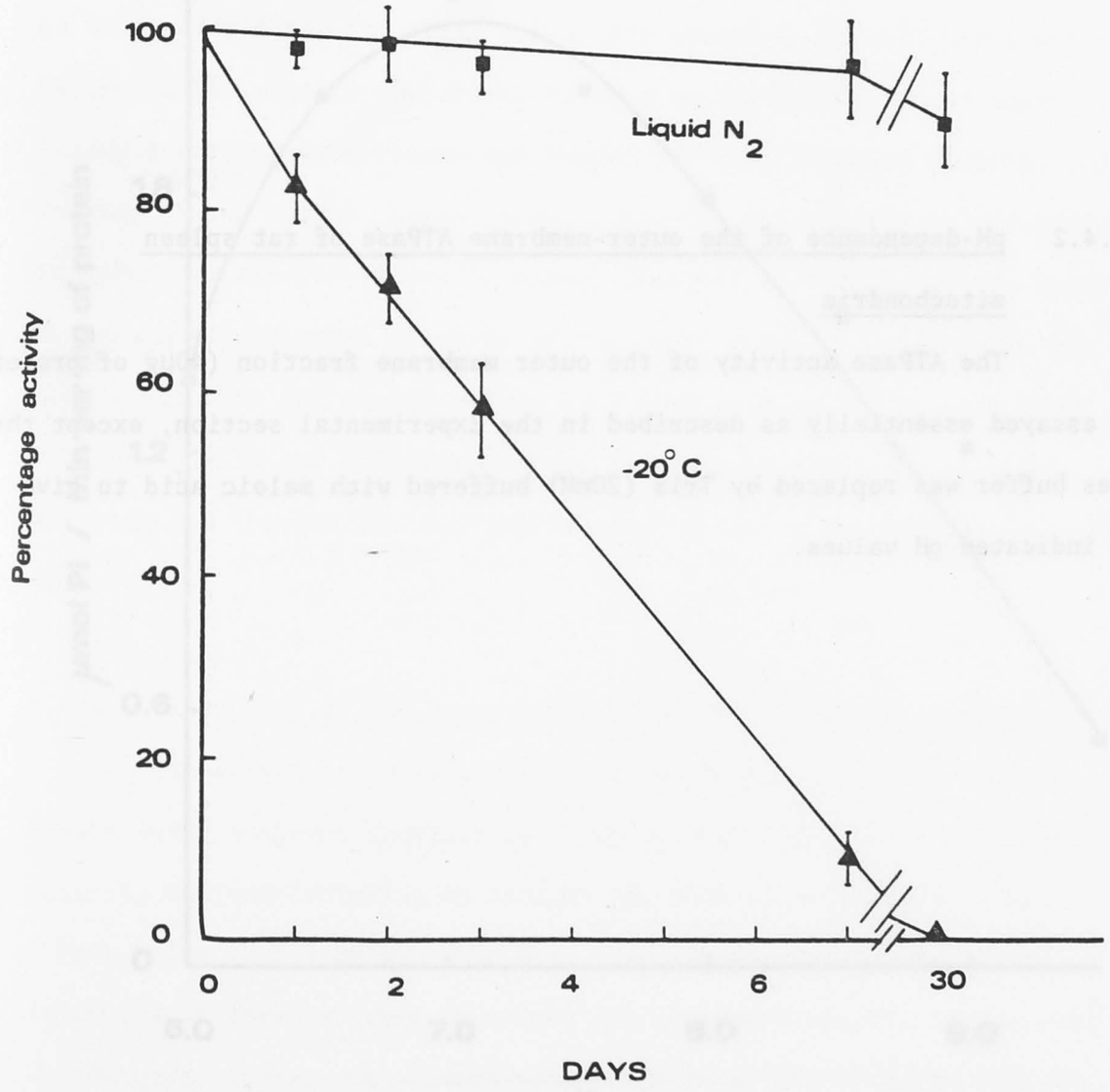
Rat spleen mitochondrial respiration was monitored, using a Clarke-type oxygen electrode, in the reaction medium described in Chapter II except that it contained, additionally, ATP (1.0mM) and succinate (2.0mM). Mitochondrial respiration was stimulated by adding Mg^{2+} (150μM) and this stimulation was taken as an indication of the presence of the Mg^{2+}-dependent ATPase activity of the outer membranes. Various modulatory metabolites and cytoplasmic extracts were added at the concentrations indicated in the Legends. "Cytoplasmic extract" represents the 650 × g supernatant fraction of rat spleen tissue (1 g wet weight) that was homogenised in 3 volumes (mls) of isolation medium (sucrose, 250mM; Hepes, 10mM, pH 7.4; EGTA, 1.0mM and bovine serum albumin, 1% w/v).

4.3.7 Calculation of free metal ion concentrations

When Mg^{2+}-EDTA (1.0mM) and Ca^{2+}-EGTA (1.0mM) buffers were used, the free metal ion concentrations in the reaction mixture were calculated as described in Chapter II. Similarly, when Ca^{2+}-nitrilotriacetate (3.0mM) buffers were used, the following dissociation constants for nitrilotriacetate.H_3 (K_1=1.89; K_2=2.49 and K_3=9.73) and the following stability constants for Ca^{2+}-nitrilotriacetate (K_4=6.46) (Sillen and Martel, 1971) were used in making the calculations.

Fig.4.1 Stability of rat spleen mitochondrial outer-membrane ATPase during storage

The mitochondrial outer-membrane preparation was stored either in liquid N_2 or at -20°C, and a small portion was removed after different storage times. The ATPase activity was assayed as described in the Experimental section, by using 40µg of membrane protein. The results represent means ± S.E.M. (bars) of triplicate determinations.


100
80
60
40
20
0
Percentage activity
Liquid N_2
-20° C
0
2
4
6
30
DAYS

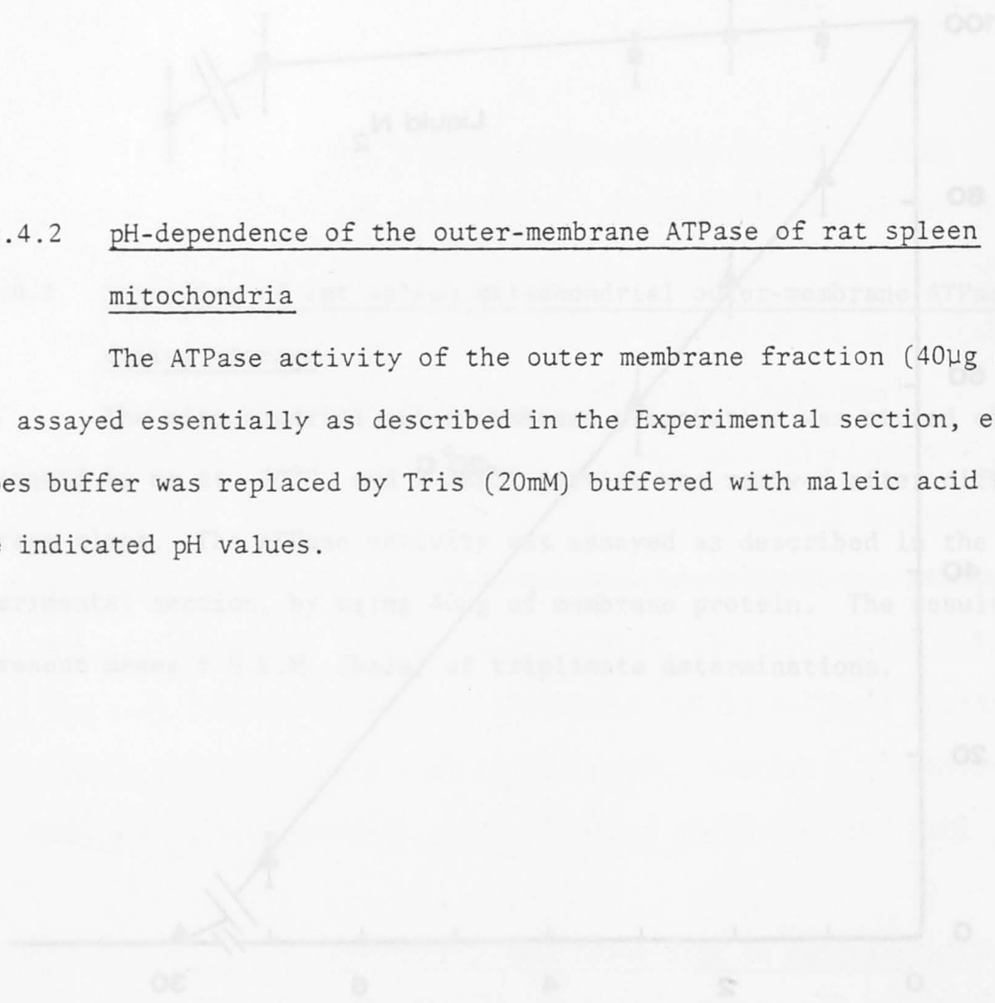
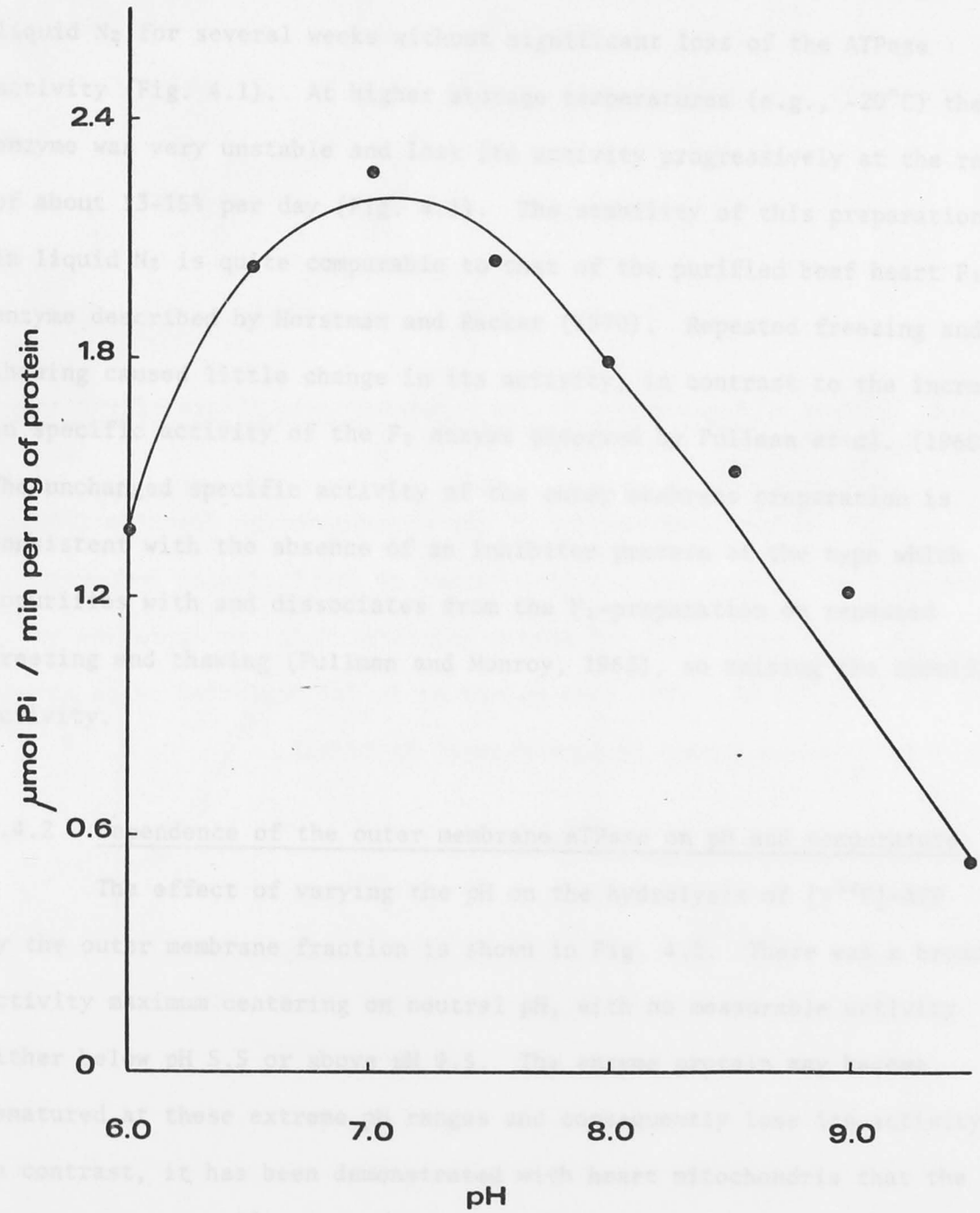


Fig.4.2 pH-dependence of the outer-membrane ATPase of rat spleen mitochondria

The ATPase activity of the outer membrane fraction (40µg of protein) was assayed essentially as described in the Experimental section, except that Hepes buffer was replaced by Tris (20mM) buffered with maleic acid to give the indicated pH values.


2.4
1.8
1.2
0.6
0
µmol Pi / min per mg of protein
6.0
7.0
8.0
9.0
pH

4.4 RESULTS

4.4.1 Stability of the outer membrane ATPase during storage

Isolated outer membranes from rat spleen mitochondria suspended in Hepes buffer (50mM adjusted to pH 7.4 with KOH) can be stored in liquid N_2 for several weeks without significant loss of the ATPase activity (Fig. 4.1). At higher storage temperatures (e.g., -20°C) the enzyme was very unstable and lost its activity progressively at the rate of about 13-15% per day (Fig. 4.1). The stability of this preparation in liquid N_2 is quite comparable to that of the purified beef heart F_1 enzyme described by Horstman and Racker (1970). Repeated freezing and thawing caused little change in its activity, in contrast to the increase in specific activity of the F_1 enzyme observed by Pullman *et al.* (1960). The unchanged specific activity of the outer membrane preparation is consistent with the absence of an inhibitor protein of the type which copurifies with and dissociates from the F_1-preparation on repeated freezing and thawing (Pullman and Monroy, 1963), so raising the specific activity.

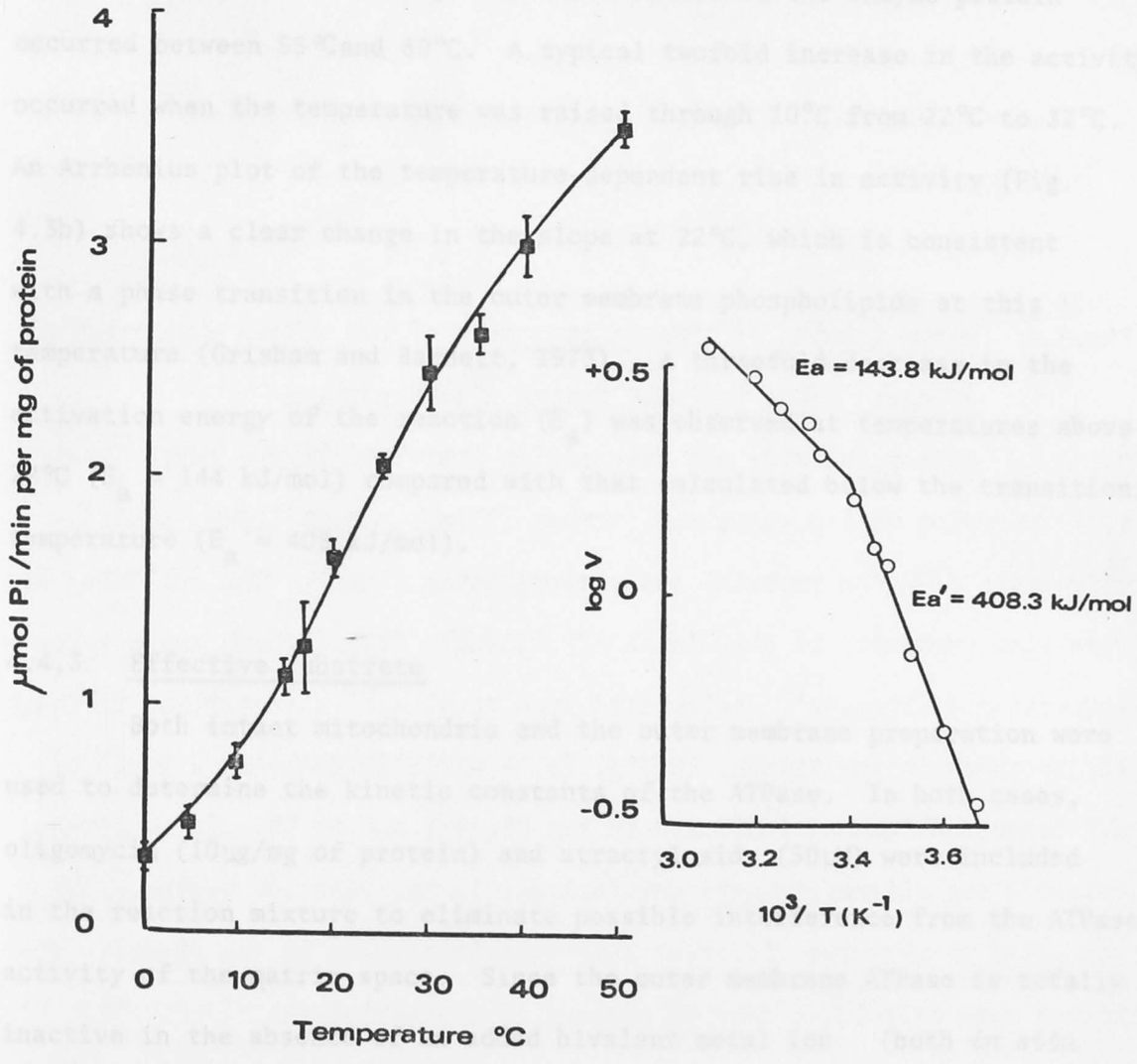
4.4.2 Dependence of the outer membrane ATPase on pH and temperature

The effect of varying the pH on the hydrolysis of [γ^{32}P]-ATP by the outer membrane fraction is shown in Fig. 4.2. There was a broad activity maximum centering on neutral pH, with no measurable activity either below pH 5.5 or above pH 9.5. The enzyme protein may become denatured at these extreme pH ranges and consequently lose its activity. In contrast, it has been demonstrated with heart mitochondria that the activity of the Mg^{2+}-stimulated external ATPase increases in response to increasing pH (in the range of pH 7.0 to pH 10) (Chao and Davis, 1972). It was suggested that the reaction buffer has more capacity to neutralise

Fig.4.3 Effect of temperature on the outer-membrane ATPase of rat spleen mitochondria

(a) ATPase activity (v) of the outer-membrane fraction (50µg of protein) was assayed after preincubation at the indicated temperatures for 10 min. The results of triplicate determinations are expressed as means ± S.E.M. (bars). (b) Same values as in (a) replotted as an Arrhenius plot with E_a values (given in parentheses) in kJ/mol.

(a) (b)


µmol Pi /min per mg of protein
0
1
2
3
4
0
10
20
30
40
50
Temperature °C
log V
+0.5
0
-0.5
Ea = 143.8 kJ/mol
Ea' = 408.3 kJ/mol
3.0
3.2
3.4
3.6
10³/ T(K⁻¹)

the protons (H^+) that are liberated from the hydrolysis of ATP in the higher pH ranges (above pH 8.0) and is therefore capable of enhancing the activity (Chao and Davis, 1972).

The activity of the enzyme responded sigmoidally to increasing temperature between 0°C and 55°C (Fig. 4.3a). Complete inactivation was observed at 60°C, indicating that denaturation of the enzyme protein occurred between 55°Cand 60°C. A typical twofold increase in the activity occurred when the temperature was raised through 10°C from 22°C to 32°C. An Arrhenius plot of the temperature-dependent rise in activity (Fig. 4.3b) shows a clear change in the slope at 22°C, which is consistent with a phase transition in the outer membrane phospholipids at this temperature (Grisham and Barnett, 1973). A threefold decrease in the activation energy of the reaction (E_a) was observed at temperatures above 22°C (E_a = 144 kJ/mol) compared with that calculated below the transition temperature (E_a = 408 kJ/mol).

4.4.3 Effective substrate

Both intact mitochondria and the outer membrane preparation were used to determine the kinetic constants of the ATPase. In both cases, oligomycin (10μg/mg of protein) and atractyloside (50μM) were included in the reaction mixture to eliminate possible interference from the ATPase activity of the matrix space. Since the outer membrane ATPase is totally inactive in the absence of an added bivalent metal ion (both *in situ* and in the outer membrane preparation), its natural substrate must be a nucleoside triphosphate-bivalent metal ion complex. When intact mitochondria were used, the ATP concentration was fixed at 300μM, and the enzyme responded to variation in free Mg^{2+} concentration in a hyperbolic manner to give a K_m value for Mg^{2+} of approximately 50μM (Fig. 4.4a). Similarly, when the free Mg^{2+} concentration was fixed at 3.0mM, variation in the ATP concentration gave a K_m value for ATP that was practically

Fig.4.4 Determination of kinetic constants of rat spleen mitochondrial ATPases, using isolated intact mitochondria

ATPase activity was measured as described in the text. The basic reaction medium contained sucrose (250mM), Hepes (25mM; pH 7.4), oligomycin (10µg/mg protein) and atractyloside (50µM). The following agents were also present: in Fig. (a) - ATP (300µM), EDTA (1.0mM) and a range of concentrations of free Mg^{2+} (calculated from total Mg^{2+} as described in Chapter II) as shown in the Figure; in Fig. (b) - $MgCl_2$ (3.0mM) and a range of ATP concentrations as indicated in the Figure; and in Fig. (c) - atractyloside and oligomycin were not included in the reaction medium. ATP was added to give the different concentrations shown in the Figure. The values were calculated from an average of 3 experiments.

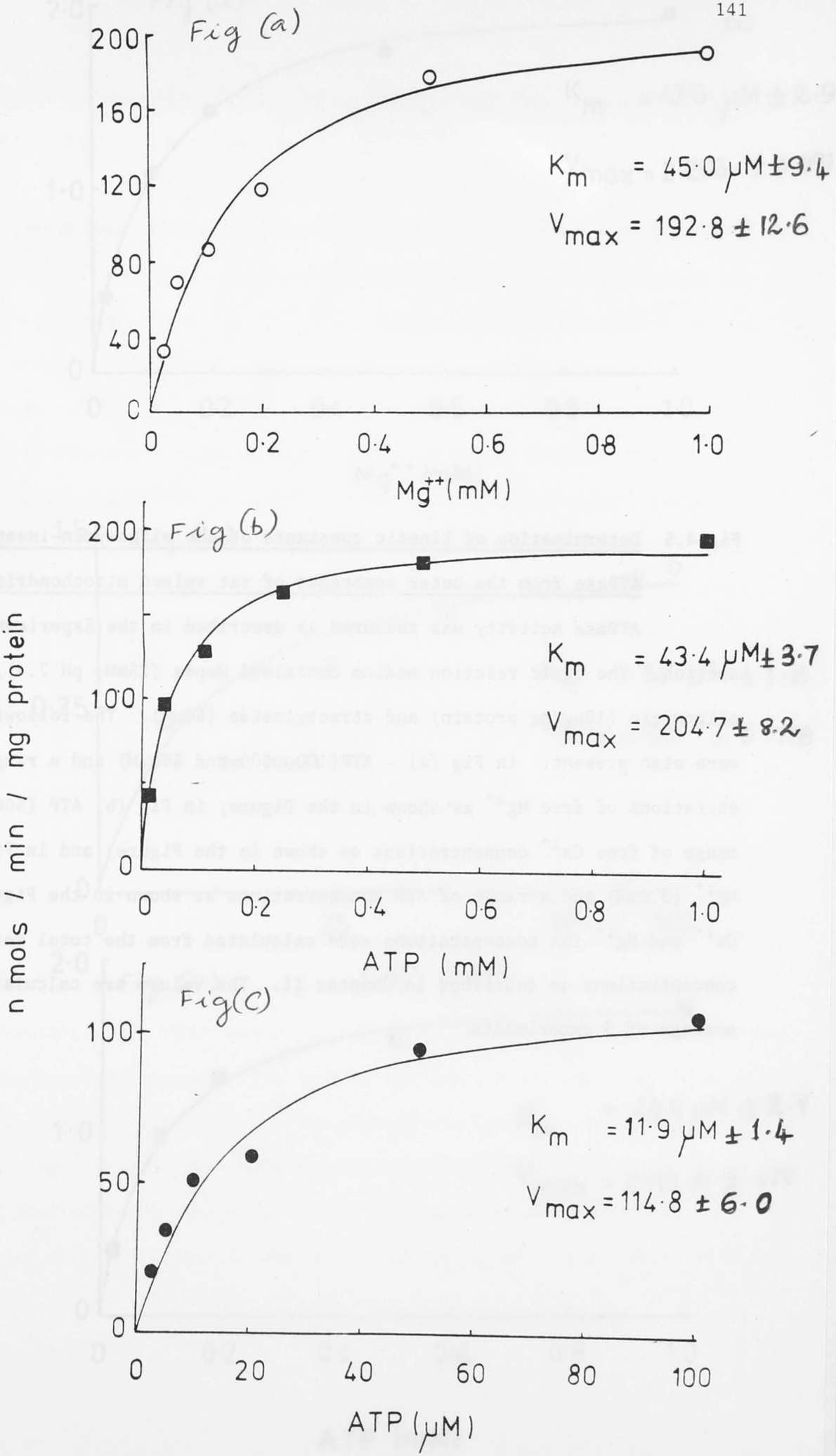


Fig (a)
200
160
120
80
40
0
0
0·2
0·4
0·6
0·8
1·0
Mg^{++} (mM)
K_m = 45·0 μM ± 9·4
V_{max} = 192·8 ± 12·6
Fig (b)
200
100
0
0
0·2
0·4
0·6
0·8
1·0
ATP (mM)
K_m = 43·4 μM ± 3·7
V_{max} = 204·7 ± 8·2
n mols / min / mg protein
Fig(C)
100
50
0
0
20
40
60
80
100
ATP (μM)
K_m = 11·9 μM ± 1·4
V_{max} = 114·8 ± 6·0

Fig.4.5 Determination of kinetic constants of the oligomycin-insensitive ATPase from the outer membranes of rat spleen mitochondria

ATPase activity was measured as described in the Experimental section. The basic reaction medium contained Hepes (25mM; pH 7.4), oligomycin (10µg/mg protein) and atractyloside (50µM). The following agents were also present: in Fig (a) - ATP(fixed at 100µM) and a range of concentrations of free Mg^{2+} as shown in the Figure; in Fig (b) ATP (500µM) and a range of free Ca^{2+} concentrations as shown in the Figure; and in Fig (c) - Mg^{2+} (3.0mM) and a range of ATP concentrations as shown in the Figure. Free Ca^{2+} and Mg^{2+} ion concentrations were calculated from the total metal ion concentrations as described in Chapter II. The values are calculated from an average of 3 experiments.

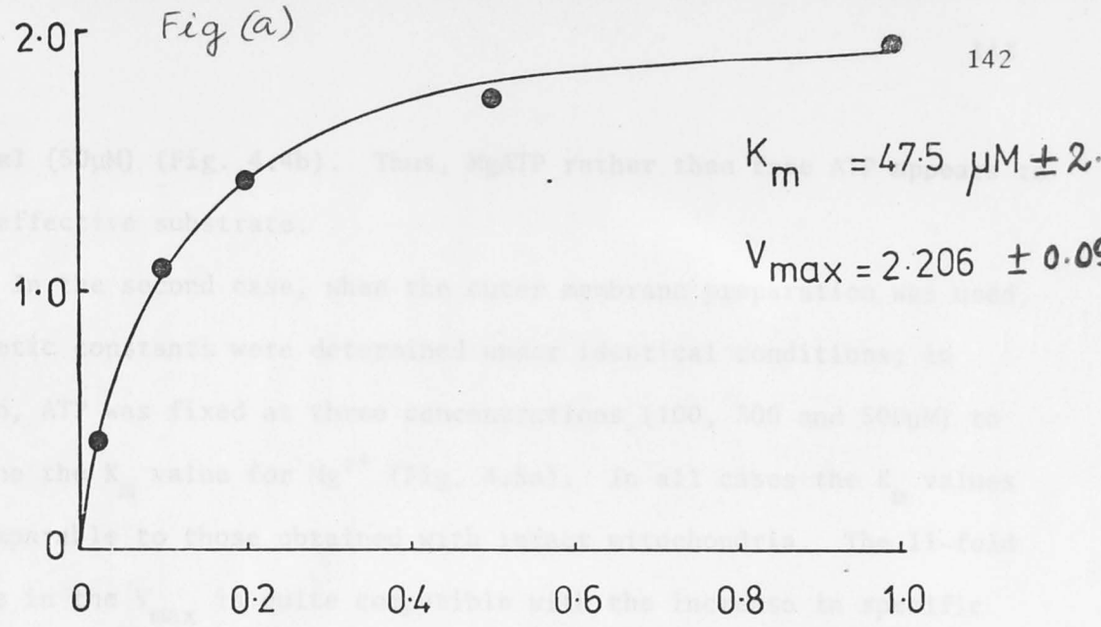


Fig (a)
2·0
1·0
0
0
0·2
0·4
0·6
0·8
1·0
K_m = 47·5 μM ± 2·9
V_{max} = 2·206 ± 0·091
Mg^{++} (mM)

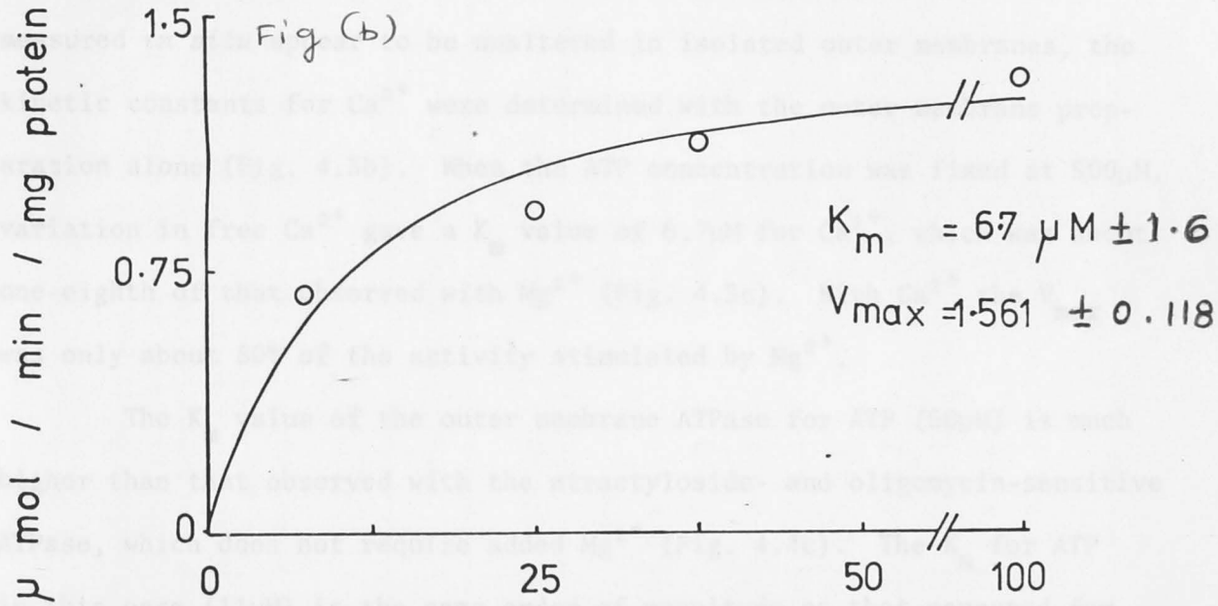


Fig (b)
1·5
0·75
0
0
25
50
100
μ mol / min / mg protein
K_m = 6·7 μM ± 1·6
V_{max} = 1·561 ± 0·118
Ca^{++} (μM)

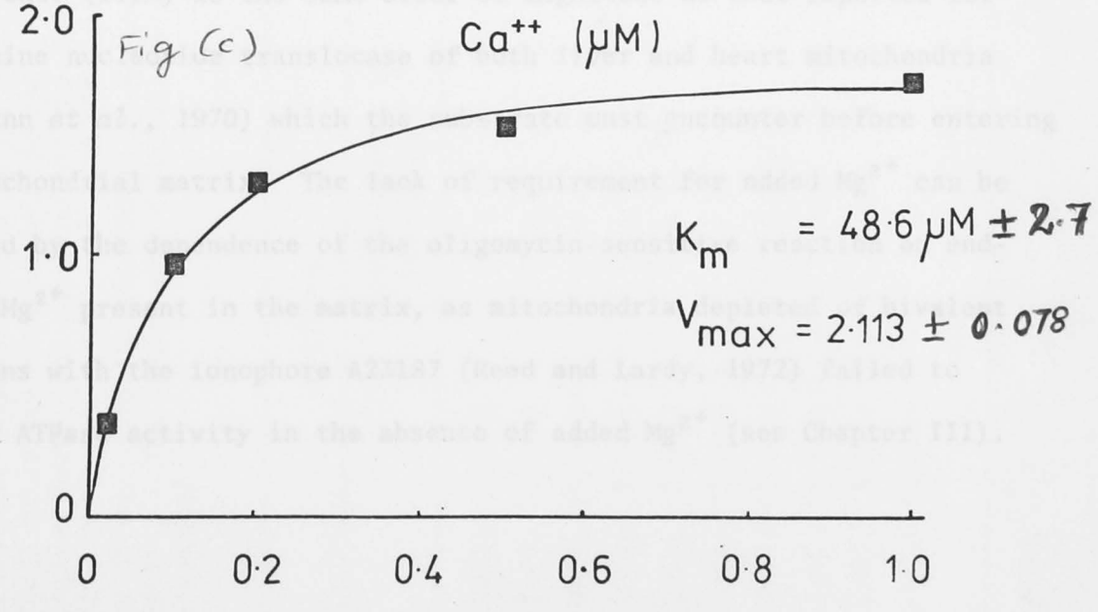


Fig (c)
2·0
1·0
0
0
0·2
0·4
0·6
0·8
1·0
K_m = 48·6 μM ± 2·7
V_{max} = 2·113 ± 0·078
ATP (mM)

identical (50μM) (Fig. 4.4b). Thus, MgATP rather than free ATP appears to be the effective substrate.

In the second case, when the outer membrane preparation was used, the kinetic constants were determined under identical conditions; in addition, ATP was fixed at three concentrations (100, 300 and 500μM) to determine the K_m value for Mg^{2+} (Fig. 4.5a). In all cases the K_m values were comparable to those obtained with intact mitochondria. The 11-fold increase in the V_{max} is quite compatible with the increase in specific activity observed with outer membrane preparations (Chapter III; Vijayakumar and Weidemann, 1976). Because the basic kinetic constants measured *in situ* appear to be unaltered in isolated outer membranes, the kinetic constants for Ca^{2+} were determined with the outer membrane preparation alone (Fig. 4.5b). When the ATP concentration was fixed at 500μM, variation in free Ca^{2+} gave a K_m value of 6.7μM for Ca^{2+}, which was about one-eighth of that observed with Mg^{2+} (Fig. 4.5c). With Ca^{2+} the V_{max} was only about 80% of the activity stimulated by Mg^{2+}.

The K_m value of the outer membrane ATPase for ATP (50μM) is much higher than that observed with the atractyloside- and oligomycin-sensitive ATPase, which does not require added Mg^{2+} (Fig. 4.4c). The K_m for ATP in this case (11μM) is the same order of magnitude as that reported for the adenine nucleotide translocase of both liver and heart mitochondria (Weidemann *et al.*, 1970) which the substrate must encounter before entering the mitochondrial matrix. The lack of requirement for added Mg^{2+} can be explained by the dependence of the oligomycin-sensitive reaction on endogenous Mg^{2+} present in the matrix, as mitochondria depleted of bivalent metal ions with the ionophore A23187 (Reed and Lardy, 1972) failed to show any ATPase activity in the absence of added Mg^{2+} (see Chapter III).

Table 4.1

Effect of bivalent metal ions, in the presence and absence of Mg^{2+}, on outer membrane ATPase activity

ATPase activity was assayed in the reaction medium described in the text, by using either the outer membrane preparation (40µg) or intact mitochondria (100µg). $MgCl_2$ was excluded from the medium when the specificity of the enzyme for bivalent metal ions was studied; the calcium concentration of the reaction mixture when reagent grade salts of Mg^{2+}, Mn^{2+}, Cu^{2+}, Co^{2+}, Sr^{2+}, Ba^{2+} and Ni^{2+} were present was estimated by atomic absorption spectrophotometry (Pybus, 1969). The ATPase activity stimulated by various bivalent metal ions is expressed as a percentage of the Mg^{2+}-stimulated activity. The values were calculated from an average of 4 experiments and are given as averages ± S.E.M. n.d. = not determined.

ADDITIONS		INTACT MITOCHONDRIA		OUTER MEMBRANE FRACTION	
	Calcium concn. of medium (µM)	No addition	Plus $MgCl_2$ (5.0mM)	No addition	Plus $MgCl_2$ (5.0mM)
$MgCl_2$ (5.0mM)	1.05	100	89.5 ± 7.2	100	93.7 ± 1.6
$CaCl_2$ (3.0mM)	3000.00	88.0 ± 4.7	96.8 ± 5.4	78.5 ± 1.9	70.2 ± 3.9
$MnCl_2$ (3.0mM)	0.90	53.9 ± 2.3	59.1 ± 3.1	51.7 ± 1.5	46.7 ± 1.4
$CuCl_2$ (3.0mM)	0.54	32.0 ± 4.8	29.3 ± 4.1	29.3 ± 4.1	32.1 ± 6.4
$CoCl_2$ (3.0mM)	0.96	24.9 ± 6.4	28.1 ± 2.8	26.2 ± 1.5	26.6 ± 2.3
$SrCl_2$ (3.0mM)	1.41	16.1 ± 3.1	101.4 ± 7.8	22.8 ± 1.8	84.8 ± 3.3
$BaCl_2$ (3.0mM)	0.72	13.5 ± 2.5	102.9 ± 6.9	20.2 ± 0.9	86.3 ± 8.1
$ZnCl_2$ (3.0mM)	nd	11.4 ± 1.7	20.2 ± 1.9	15.6 ± 0.6	23.7 ± 1.4
$CdCl_2$ (3.0mM)	nd	16.6 ± 3.6	18.9 ± 3.1	14.4 ± 2.4	19.1 ± 5.3
$NiCl_2$ (3.0mM)	1.26	10.1 ± 2.1	17.1 ± 3.2	10.2 ± 1.0	15.7 ± 1.8

Table 4.2

Ability of various nucleoside triphosphates to replace ATP as substrate for the rat spleen mitochondrial outer membrane ATPase

ATPase activity of the mitochondrial outer membrane preparation (50µg of protein) was assayed as described in the text. $MgCl_2$ (4.0mM) and [γ-^{32}P] ATP (0.1mM; 1.5×10^5 d.p.m.) were added uniformly in all incubations. All values where unlabelled nucleoside triphosphates (1.0mM) were added are expressed as a percentage of the ^{32}P released at saturating concentrations of [γ-^{32}P] ATP (0.1mM). The values are from an average of 3 experiments and are given as averages ± S.E.M.

ADDITIONS	^{32}P LIBERATED (%)
None	100
ATP	33.9 ± 3.1
GTP	89.9 ± 6.4
UTP	86.9 ± 4.7
CTP	87.7 ± 2.9
ITP	69.2 ± 3.2

4.4.4 Bivalent-metal-ion specificity

A spectrum of bivalent metal ions was used to test (i) their ability to support ATP hydrolysis independently of Mg^{2+} and (ii) their ability to modify the Mg^{2+}-stimulated ATPase activity (Table 4.1). Mg^{2+} was the most effective metal-ion activator, although it could be replaced by Ca^{2+} with only 20-30% loss of activity. Mn^{2+} alone was 50% less effective than Mg^{2+}, whereas Co^{2+}, Sr^{2+}, Ba^{2+}, Zn^{2+}, Cd^{2+} and Ni^{2+}, in decreasing order of efficiency, supported very little ATP hydrolysis. The small amount of hydrolysis found with these ions can mostly be accounted for by the presence of Ca^{2+}, for which the enzyme has a very low K_m (Fig. 4.5b), as a contaminating ion in the analytical reagent grade salts used (Table 4.1). The most striking observation was made with Cu^{2+}, which supported ATP hydrolysis at high concentrations (3.0mM) but was a very effective inhibitor at low concentrations (see Fig. 4.7). The bivalent metal ion dependence was almost identical whether the experiments were conducted *in situ* or with the outer-membrane preparation. Ca^{2+}, however, inhibited the Mg^{2+}-stimulated ATPase activity more strongly when the outer membrane preparation was used.

4.4.5 Nucleoside triphosphate specificity

To determine the specificity of the nucleoside triphosphates, the liberation of $^{32}P_i$ from [γ-^{32}P] ATP added at saturating concentration (100μM) was measured in the presence of a tenfold excess (1.0mM) of either unlabelled ATP or one of several competing unlabelled nucleoside triphosphates (Table 4.2). The specificity of the reaction for ATP was shown clearly by the strong inhibition of $^{32}P_i$ release by unlabelled ATP (a decrease to only 34% of the control rate). Of the other unlabelled nucleoside triphosphates tested, only ITP produced a significant diminution in $^{32}P_i$ release, decreasing the rate to 69% of the control value. Thus, ITP is able to replace ATP with approximately 50% efficiency.

Table 4.3

Effect of various glycolytic intermediates on Mg^{2+}-stimulated state-3 respiration by rat spleen mitochondria

Mg^{2+}-stimulated respiration by rat spleen mitochondria was measured as described in the text, and was taken as 100%. Various glycolytic intermediates were added at the concentrations indicated, and the new rate of respiration was expressed as the percentage of Mg^{2+}-stimulated activity; where applicable, the cytoplasmic extract (100µl) was added before the rate of respiration was measured. The values are averages of duplicate experiments.

ADDITION		ACTIVITY (%)	
		Cytoplasmic Extract (-)	(+)
Mg^{2+}		100.0	102.1
glucose	(5 mM)	98.7	87.6
glucose-6 phosphate	(1.5mM)	106.8	114.5
fructose-6 phosphate	(3 mM)	101.2	107.3
fructose-diphosphate	(0.5mM)	114.4	118.3
3-phosphoglycerate	(2 mM)	128.0	121.2
2:3 diphospho glycerate	(2 mM)	126.2	127.0
dihydroxy acetone phosphate	(2 mM)	129.2	138.7
glyceraldehyde diphosphate	(2 mM)	106.4	111.2
phosphoenol-pyruvate	(2 mM)	93.3	87.6
lactate	(3 mM)	84.4	87.2

4.4.6 Influence of possible effector moleçules on the Mg^{2+}-stimulated respiration (external ATPase activity) of rat spleen mitochondria

In the design of this experiment, rat spleen mitochondrial respiration was monitored as described in the Experimental section, in a reaction medium containing a respiratory substrate (e.g., succinate, 2mM) and ATP (1mM). The state-3 respiration was stimulated by adding Mg^{2+} (>150μM), and the stimulation was taken to be a measure of the ATPase activity of the outer membranes. This assumption is based on the observation that Mg^{2+} stimulates the outer membrane ATPase in the presence of ATP, giving rise to ADP which, in turn, stimulates state-3 respiration. A variety of effector molecules was tested in an attempt to scan several chemically- or metabolically-related groups of molecules, which either have been reported to be effectors of ATPases or for which some regulatory role might be expected on teleological grounds. The concentrations used were not intended to approximate physiological levels, but were high enough to ensure that any potential effect would be seen. The effect of a cytoplasmic extract (100μl) on the state-3 respiration was also tested in the presence of the ligands listed below, as the expression of modulatory activity might require the presence of a cytoplasmic protein component to act as the actual ligand-induced activator or inhibitor.

The ability of various glycolytic intermediates to modulate the Mg^{2+}-stimulated state-3 respiration is shown in Table 4.3. Of the intermediates tested, the triose phosphates (e.g., 3-phosphoglycerate, dihydroxyacetone phosphate, etc.) appeared to be mild activators, whilst phospoenol-pyruvate and lactate were mild inhibitors. The other glycolytic intermediates tested had no significant effect. The cytoplasmic extract had no significant additional effect although, in its presence, glucose-6 phosphate became slightly stimulatory.

Table 4.4

Effect of various tricarboxylic acid cycle intermediates on the regulation of Mg^{2+}-stimulated state-3 respiration by rat spleen mitochondria

Conditions for the measurement of mitochondrial respiration are given in the Table 4.3. All values are expressed as a percentage of Mg^{2+}-stimulated respiration.

ADDITION		ACTIVITY (%)	
		Cytoplasmic Extract (-)	(+)
pyruvate	(3.0mM)	113.6	126.4
α-oxoglutarate	(3.0mM)	122.3	131.7
citrate	(3.0mM)	108.7	116.4
succinate	(2.0mM)	102.9	107.0
fumerate	(2.5mM)	102.0	114.8
malate	(3.0mM)	106.2	103.0
oxalacetate	(3.0mM)	98.9	97.2
acetyl-CoA	(2.0mM)	116.4	113.8
coenzyme-A	(1.0mM)	106.3	112.6

Table 4.5

Effect of amino acids in the modulation of Mg^{2+}-stimulated state-3 respiration by rat spleen mitochondria

Conditions for the measurement of mitochondrial respiration are given in the Table 4.3. All values are expressed as a percentage of Mg^{2+}-stimulated respiration.

ADDITION		ACTIVITY (%)	
		Cytoplasmic Extract (-)	(+)
alanine	(2.5mM)	85.5	85.3
arginine	(2.5mM)	92.6	107.4
glutamine	(2.5mM)	112.8	123.4
glutamic acid	(3.0mM)	109.4	120.6
aspartate	(3.0mM)	110.5	111.3
cystine (C-S-S-C)	(3.0mM)	97.2	93.7

Table 4.6

Effect of a variety of possible metabolic intermediates on the modulation of Mg^{2+}-stimulated state-3 respiration by rat spleen mitochondria

Conditions for the measurement of mitochondrial respiration are as given in the Table 4.3. All values are expressed as a percentage of Mg^{2+}-stimulated respiration.

ADDITION		ACTIVITY (%)	
		Cytoplasmic Extract (-)	(+)
3-hydroxybutyrate	(3.0mM)	110.0	107.2
creatine phosphate	(2.0mM)	114.9	104.2
creatine	(2.0mM)	108.0	107.8
potassium acetate	(2.0mM)	121.1	117.6
carnitine	(2.0mM)	111.0	116.3
glutathione			
(a) reduced	(1.0mM)	108.2	118.6
(b) oxidised	(1.0mM)	116.1	115.5
cyclic AMP	(1.0mM)	122.0	108.7
cyclic GMP	(0.5mM)	107.6	108.7
AMP	(1.0mM)	126.7	133.2
GDP	(1.0mM)	112.0	116.1
IDP	(1.0mM)	97.8	107.8
adenine	(2.5mM)	81.1	85.8
adenosine	(2.5mM)	83.5	86.1
xanthine	(2.5mM)	95.8	104.1
hypoxanthine	(2.5mM)	93.7	101.6

When various tricarboxylic acid cycle intermediates were tested, the two α-oxo acids (pyruvate and α-oxo glutarate), citrate and acetyl-CoA stimulated the activity moderately (10-25%) whilst the other intermediates were without significant effect (Table 4.4). The cytoplasmic extract had no influence on the rates of respiration observed with any of these intermediates.

Table 4.5 shows that none of the amino acids tested, with the exception of glutamine, glutamate and aspartate, which caused a moderate stimulation (about 10-20%), were able to influence the Mg^{2+}-stimulated state-3 respiration. A variety of other compounds (listed in Table 4.6) were without significant effect on the state-3 respiration induced by Mg^{2+}, except for a mild stimulation by carnitine, potassium acetate, cyclic AMP and 5'-AMP. An obvious explanation for the 30% increase in the state-3 respiration in the presence of AMP would be the production of ADP through the action of adenylate kinase which utilises ATP and AMP in the presence of Mg^{2+}, as shown:

$$\text{ATP} + \text{AMP} \quad \underset{\leftarrow \text{ adenylate kinase}}{\xrightarrow{Mg^{2+}}} \quad 2\ \text{ADP}$$

With the exception of adenine and adenosine, which inhibited the Mg^{2+}-induced state-3 respiration by approximately 20%, none of the substances tested inhibited the activity significantly. Obviously, this method contains many factors apart from the outer membrane ATPase that are instrumental in inducing state-3 respiration by Mg^{2+}-ions. Hence, to avoid interference from other membrane components, outer membranes were isolated from spleen mitochondria and the effects of various compounds were tested on the ATPase activity directly.

Table 4.7

Effect of monovalent cations on the outer membrane ATPase activity from rat spleen mitochondria

The ATPase activity of mitochondrial outer membranes (50µg of protein was assayed as described in the text. Where indicated, ouabain (1.0mM) and/or univalent cations, at the concentrations indicated in this Table, were present in the reaction medium. The results are expressed as a percentage of Mg^{2+}-stimulated activity, which was taken as 100%.

ADDITION		ACTIVITY (%)	
		Ouabain	
		(-)	(+)
$MgCl_2$	(5.0mM)	100	96.5
plus NaCl	(150mM)	105.7	104.5
plus KCl	(5.0mM)		
plus NaCl	(20 mM)	109.1	107.6
	(500mM)	83.2	79.8
plus KCl	(20 mM)	107.6	105.3
	(500mM)	108.4	103.2
plus LiCl	(20 mM)	93.1	101.5
	(500mM)	71.7	65.6
plus CsCl	(20 mM)	101.5	99.2
	(500mM)	74.6	69.8
plus RbCl	(20 mM)	108.8	97.7
	(500mM)	80.8	78.7
plus NH_4Cl	(20 mM)	106.1	107.9
	(500mM)	69.4	66.5

Fig.4.6 Effect of univalent cations on spleen mitochondrial outer-membrane ATPase activity

ATPase activity was assayed by using mitochondrial outer membranes (50µg of protein) as described in the text, at different concentrations of univalent cations: Na^+ (●); Na^+ + K^+ (5.0mM) (○); K^+ + Na^+ (5.0mM) (△); K^+ (▲).

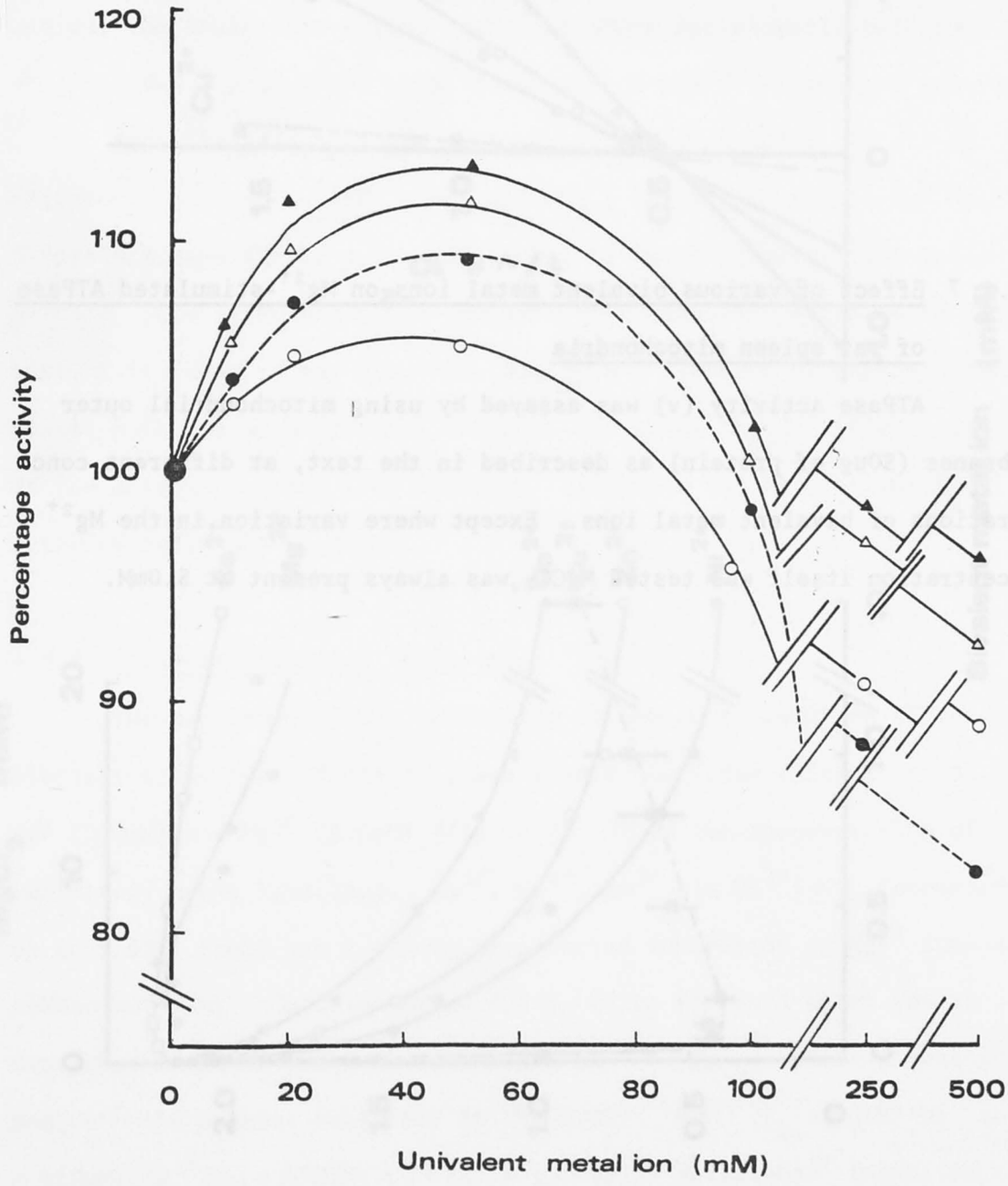
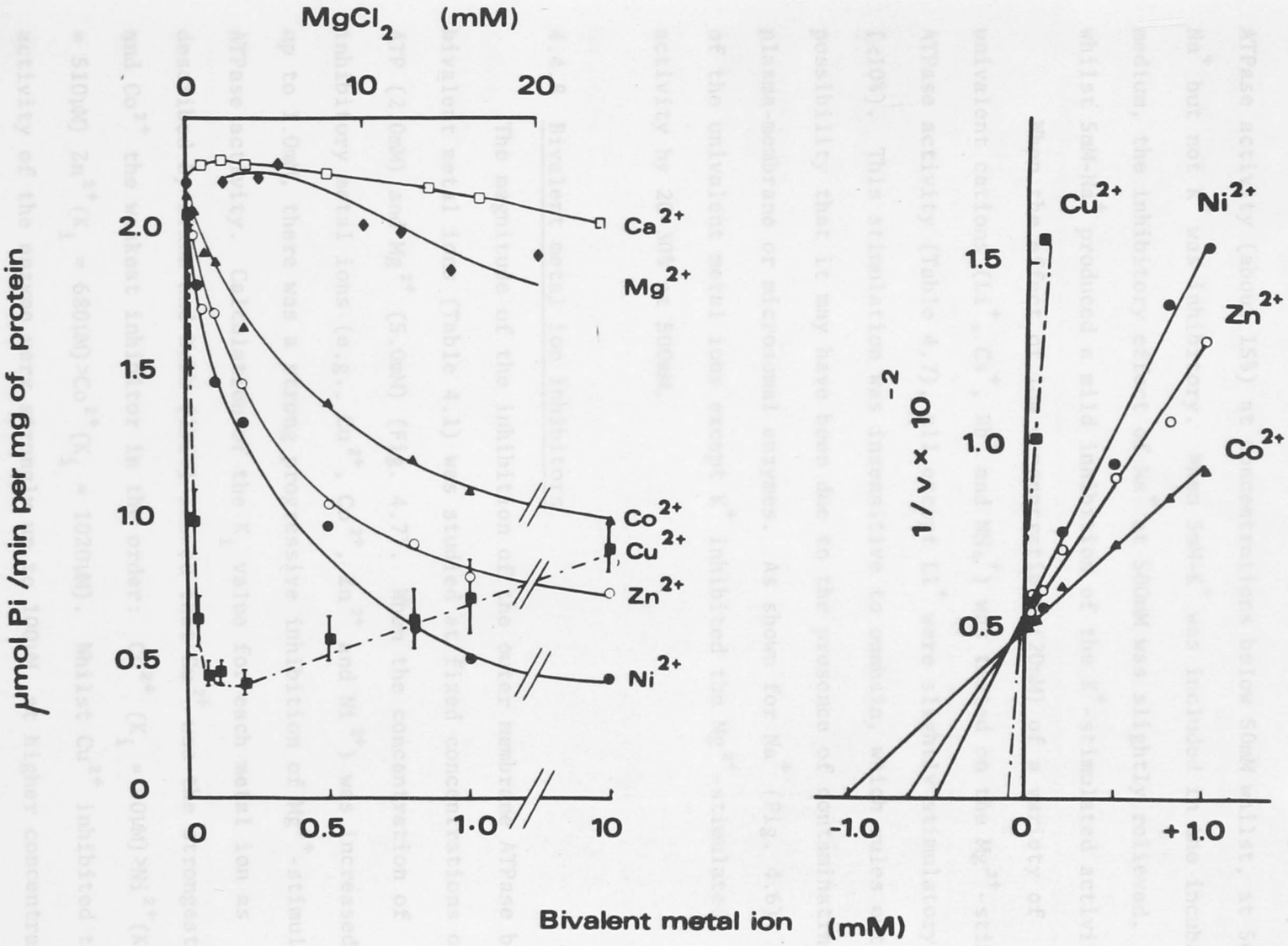

120
110
100
90
80
Percentage activity
0
20
40
60
80
100
250
500
Univalent metal ion (mM)

Fig.4.7 Effect of various bivalent metal ions on Mg^{2+}-stimulated ATPase of rat spleen mitochondria

ATPase activity (v) was assayed by using mitochondrial outer membranes (50µg of protein) as described in the text, at different concentrations of bivalent metal ions. Except where variation in the Mg^{2+} concentration itself was tested $MgCl_2$, was always present at 5.0mM.


MgCl2 (mM)
0
10
20
2.0
1.5
1.0
0.5
0
µmol Pi /min per mg of protein
Ca2+
Mg2+
Co2+
Cu2+
Zn2+
Ni2+
0
0.5
1·0
10
1 / v x 10-2
Cu2+
Ni2+
Zn2+
Co2+
1.5
1.0
0.5
-1.0
0
+1.0
Bivalent metal ion (mM)

4.4.7 Stimulation and inhibition by univalent cations

The effect of Na^+ and K^+ was tested over a wide concentration range (0-500mM) (Fig. 4.6). Both metal ions stimulated the Mg^{2+}-dependent ATPase activity (about 15%) at concentrations below 50mM whilst, at 500mM, Na^+ but not K^+ was inhibitory. When 5mM-K^+ was included in the incubation medium, the inhibitory effect of Na^+ at 500mM was slightly relieved, whilst 5mM-Na^+ produced a mild inhibition of the K^+-stimulated activity.

When the effect of low concentrations (20mM) of a variety of univalent cations (Li^+, Cs^+, Rb^+ and NH_4^+) was tested on the Mg^{2+}-stimulated ATPase activity (Table 4.7), all except Li^+ were slightly stimulatory (<10%). This stimulation was insensitive to ouabain, which rules out the possibility that it may have been due to the presence of contaminating plasma-membrane or microsomal enzymes. As shown for Na^+ (Fig. 4.6), all of the univalent metal ions except K^+ inhibited the Mg^{2+}-stimulated activity by 20-30% at 500mM.

4.4.8 Bivalent metal ion inhibitors

The magnitude of the inhibition of the outer membrane ATPase by bivalent metal ions (Table 4.1) was studied at fixed concentrations of ATP (2.0mM) and Mg^{2+} (5.0mM) (Fig. 4.7). When the concentration of inhibitory metal ions (e.g., Cu^{2+}, Co^{2+}, Zn^{2+} and Ni^{2+}) was increased up to 1.0mM, there was a strong progressive inhibition of Mg^{2+}-stimulated ATPase activity. Calculation of the K_i value for each metal ion as described by Dixon and Webb (1964) showed that Cu^{2+} was the strongest and Co^{2+} the weakest inhibitor in the order: Cu^{2+} (K_i = 90μM) > Ni^{2+} (K_i = 510μM) Zn^{2+} (K_i = 680μM) > Co^{2+} (K_i = 1020μM). Whilst Cu^{2+} inhibited the activity of the enzyme very strongly up to 100μM, at higher concentrations (up to 10mM) it also behaved as a substrate and restimulated the ATPase activity up to 25% of the uninhibited Mg^{2+}-stimulated rate. Although Mg^{2+} and Ca^{2+} were the most effective bivalent metal ion activators,

Table 4.8

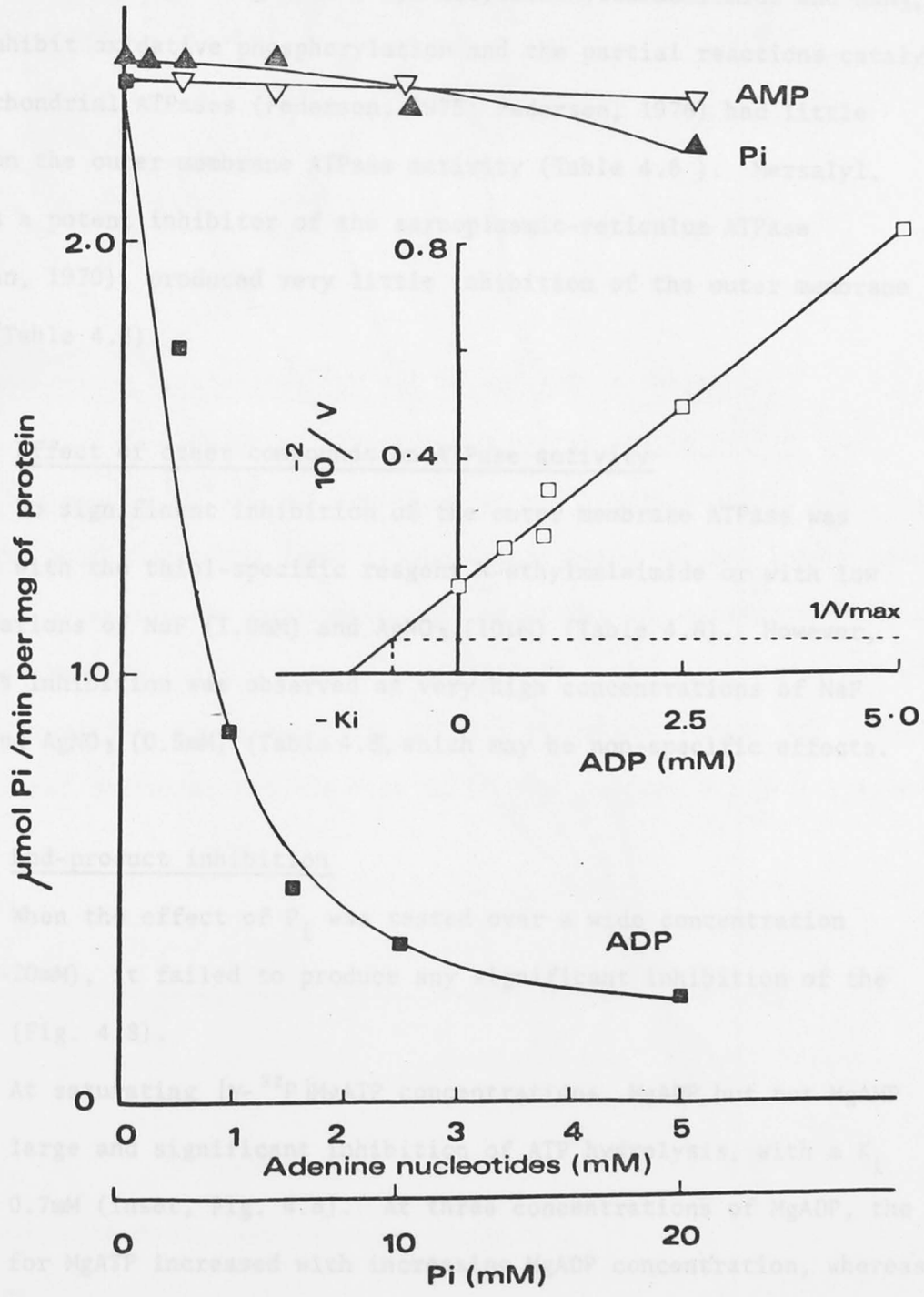
Effect of various inhibitors on spleen mitochondrial outer membrane ATPase

ATPase activity was assayed as described in the Experimental section, by using either intact mitochondria (100µg of protein) or outer membrane preparations (40µg of protein). Inhibitors were added to give the final concentrations indicated in the Table. Values are given as an average of duplicate experiments.

Additions	Concn. (mM)	ACTIVITY (%) Intact Mitochondria	Outer Membranes
$MgCl_2$	5.0	100	100
N,N'Dicyclo-hexylcarbodiimide	0.01	96.7	-
	0.10	92.9	91.7
Oligomycin (µg/mg of protein)	0.5	-	95.6
	5.0	98.1	97.2
	10.0	92.7	91.4
NaN_3	0.01	91.8	91.2
	0.10	89.9	84.0
Mersalyl	0.001	-	89.6
	0.005	93.7	92.4
	0.010	87.3	91.5
N-Ethyl-maleimide	0.004	88.8	96.2
	0.010	88.5	95.3
NaF	1.0	87.2	98.7
	10.0	40.8	40.2
$AgNO_3$	0.01	-	87.4
	0.10	55.2	54.5
	0.50	61.7	42.3

Fig.4.8 Influence of ADP, AMP and P_i on the ATPase activity of rat spleen mitochondrial outer membranes

ATP activity (v) was assayed in mitochondrial outer membranes (50µg of protein) as described in the text at a fixed, saturating concentration of MgATP (2.0mM), under conditions where the concentrations of ADP, AMP and P_i were varied. The inset shows the values in the presence of ADP replotted by the method of Dixon and Webb (1964).


AMP
Pi
2·0
1.0
0
μmol Pi /min per mg of protein
ADP
0 1 2 3 4 5
Adenine nucleotides (mM)
0 10 20
Pi (mM)
0·8
0·4
$10^{-2}/V$
1/Vmax
-Ki
0
2.5
5·0
ADP (mM)

they were slightly inhibitory in the higher concentration range (Fig. 4.7).

4.4.9 "Membrane-bound ATPase" inhibitors

The inhibitors oligomycin, N,N'dicyclohexylcarbodiimide and NaN_3, which inhibit oxidative phosphorylation and the partial reactions catalysed by mitochondrial ATPases (Pedersen, 1975; Pedersen, 1976) had little effect on the outer membrane ATPase activity (Table 4.8). Mersalyl, which is a potent inhibitor of the sarcoplasmic-reticulum ATPase MacLennan, 1970), produced very little inhibition of the outer membrane ATPase (Table 4.8).

4.4.10 Effect of other compounds on ATPase activity

No significant inhibition of the outer membrane ATPase was detected with the thiol-specific reagent N-ethylmaleimide or with low concentrations of NaF (1.0mM) and $AgNO_3$ (10μM) (Table 4.8). However, about 60% inhibition was observed at very high concentrations of NaF (10mM) and $AgNO_3$ (0.5mM) (Table 4.8), which may be non-specific effects.

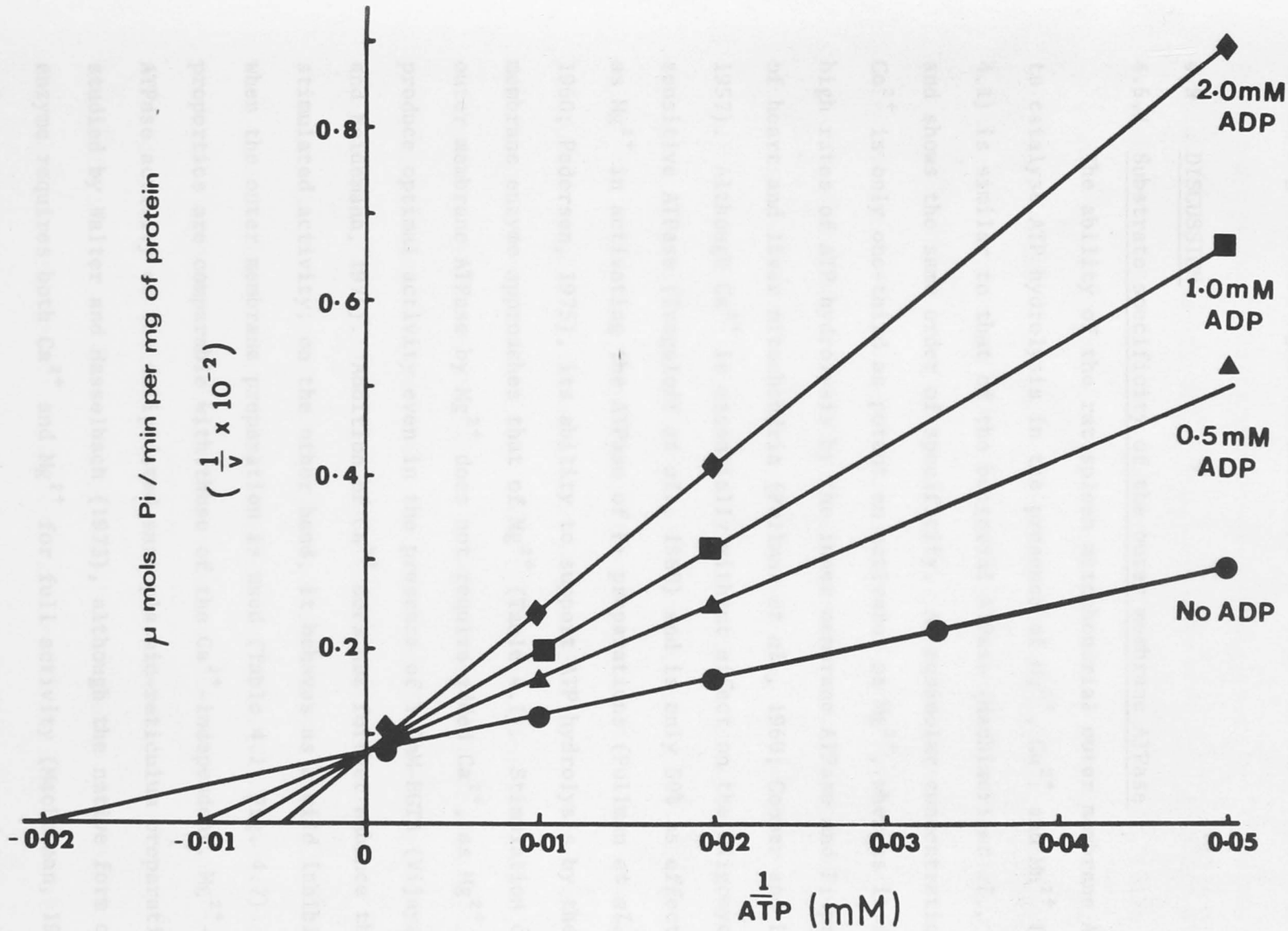
4.4.11 End-product inhibition

When the effect of P_i was tested over a wide concentration range (0-20mM), it failed to produce any significant inhibition of the reaction (Fig. 4.8).

At saturating [γ-^{32}P]MgATP concentrations, MgADP but not MgAMP caused a large and significant inhibition of ATP hydrolysis, with a K_i value of 0.7mM (inset, Fig. 4.8). At three concentrations of MgADP, the K_m value for MgATP increased with increasing MgADP concentration, whereas the V_{max} was unchanged (Fig. 4.9). This observation is consistent with the view that MgADP inhibits the enzyme by competing with MgATP directly for occupation of the catalytic site. In the absence of added MgADP, and also at all three MgADP concentrations tested, titration of the ATPase

Fig.4.9 Competitive inhibition of the Mg^{2+}-stimulated outer-membrane ATPase of rat spleen mitochondria by MgADP

ATPase activity was assayed over the [γ-^{32}P]ATP concentration range 0.01-2.0mM, by using mitochondrial outer membranes (50µg of protein). In each experiment the initial ADP concentration was set as indicated in the Fig. $MgCl_2$ was added in all cases to give a final concentration of 4.0mM. The double-reciprocal plot is expressed as the reciprocal of the reaction rate (v) against 1/[ATP] at each ADP concentration tested.


2·0mM ADP
1·0mM ADP
0·5mM ADP
No ADP
μ mols Pi / min per mg of protein
$(\frac{1}{v} \times 10^{-2})$
0·8
0·6
0·4
0·2
-0·02
-0·01
0
0·01
0·02
0·03
0·04
0·05
$\frac{1}{ATP}$ (mM)

activity with MgATP gave conventional Michaelis-Menten kinetics (Hill coefficient h = 1.0), indicating that the presence of more than one type of binding site for MgATP is unlikely.

4.5 DISCUSSION

4.5.1 Substrate specificity of the outer membrane ATPase

The ability of the rat spleen mitochondrial outer membrane ATPase to catalyse ATP hydrolysis in the presence of Mg^{2+}, Ca^{2+} and Mn^{2+} (Table 4.1) is similar to that of the bacterial ATPase (Hachimori *et al.*, 1970) and shows the same order of specificity. At equimolar concentrations, Co^{2+} is only one-third as potent an activator as Mg^{2+}, whereas it supports high rates of ATP hydrolysis by the inner membrane ATPase and F_1-preparations of heart and liver mitochondria (Pullman *et al.*, 1960; Cooper and Lehninger, 1957). Although Ca^{2+} is essentially without effect on the oligomycin-sensitive ATPase (Tzagoloff *et al.*, 1968) and is only 50% as effective as Mg^{2+} in activating the ATPase of F_1 preparations (Pullman *et al.*, 1960; Pedersen, 1975), its ability to support ATP hydrolysis by the outer membrane enzyme approaches that of Mg^{2+} (Table 4.1). Stimulation of the outer membrane ATPase by Mg^{2+} does not require added Ca^{2+}, as Mg^{2+} can produce optimal activity even in the presence of 1.0mM-EGTA (Vijayakumar and Weidemann, 1976). Addition of Ca^{2+} does not further enhance the Mg^{2+}-stimulated activity; on the other hand, it behaves as a mild inhibitor when the outer membrane preparation is used (Table 4.1; Fig. 4.7). These properties are comparable with those of the Ca^{2+}-independent, Mg^{2+}-stimulated ATPase activity of the delipidated sarcoplasmic-reticulum preparation studied by Walter and Hasselbach (1973), although the native form of that enzyme requires both Ca^{2+} and Mg^{2+} for full activity (MacLennan, 1970).

The difference in effect of the various bivalent metal ions on Mg^{2+}-stimulated ATP hydrolysis can be explained in terms of their affinity for ATP. Cu^{2+} for instance, binds ATP 120 times as strongly as Mg^{2+} (Sillén and Martell, 1971) and effectively displaces Mg^{2+} from the MgATP complex, which explains the strong inhibition when Cu^{2+} is added at much lower concentration (<100μM) than Mg^{2+} (5.0mM) (Fig. 4.7). Similarly, Zn^{2+}, Ni^{2+} and Co^{2+} have 3-6 times higher affinity for ATP than Mg^{2+} (Sillén and Martell, 1971), which is consistent with their ability to inhibit Mg^{2+}-stimulated ATP hydrolysis (Fig. 4.7). The inability of Ca^{2+}, Sr^{2+} and Ba^{2+} to inhibit the Mg^{2+}-stimulated ATPase activity in intact mitochondria (Table 4.1) is compatible with their lower affinity for ATP compared with Mg^{2+} (Sillén and Martell, 1971). The response of the outer membrane ATPase to bivalent metal ions in direct proportion to their relative ability to bind ATP supports the view that the effective substrate is a bivalent metal ion-nucleoside triphosphate complex.

The nucleoside triphosphate specificity of the enzyme (Table 4.2) is similar to that of the oligomycin-sensitive ATPase of heart and liver mitochondria (Tzagoloff *et al.*, 1968; Cooper and Lehninger, 1957), chloroplasts (Vambutas and Racker, 1965) and bacteria (Hanson and Kennedy, 1973) in that it catalyses the preferential hydrolysis of purine nucleoside triphosphates, with ATP being hydrolysed at about twice the rate of ITP. ITP can also partly displace the atractyloside-insensitive [^{14}C] ADP binding to ox heart mitochondria (Weidemann *et al.*, 1969), which is compatible with the presence of a purine-nucleoside-binding protein located outside the atractyloside barrier in mitochondria of heart (Chao and Davis, 1972) and spleen (Vijayakumar and Weidemann, 1976). It is suggested that the atractyloside-insensitive binding site for ITP and ADP

in these mitochondria could be the outer membrane ATPase.

The double-reciprocal plot obtained with the outer membrane ATPase of rat spleen mitochondria when the concentration of MgATP is increased, differs markedly from that observed with the rat liver mitochondrial ATPase. Since the oligomycin-sensitive ATPase of liver mitochondria has two interdependent nucleoside-binding sites (Koshland, 1970; Kitiakowsky and Rosenberg, 1952) similar to ox heart F_1 preparations (Penefsky, 1974), and a separate modifying site for anions (Alberty *et al.*, 1954; Frieden, 1964; Pedersen, 1976), it gives a curvature in the double-reciprocal plot (that becomes linear only on addition of anions) when MgATP is the sole substrate (e.g., P_i) (Ebel and Lardy, 1975). In contrast, the outer membrane ATPase gives a linear double-reciprocal plot even in the absence of added anions (Fig. 4.9), which suggests that the modifying site is absent in this case. Lack of stimulation of the outer membrane ATPase by P_i (Fig. 4.8) provides additional support for the absence of the modifying site.

4.5.2 Inhibition of the outer membrane ATPase

Competitive inhibition of the outer membrane ATPase by ADP (Fig. 4.8) is similar to that reported for F_1-preparations from chloroplasts (Vambutas and Racker, 1965) and bacteria (Schnebli and Abrams, 1970). Further, the addition of ADP increases the K_m value for MgATP without changing the V_{max} or the linearity of the double-reciprocal plot (Fig. 4.9), which suggests that this enzyme has only one nucleotide binding site for which both ADP and ATP compete. AMP does not inhibit the activity of either the outer membrane ATPase (Fig. 4.8) or the oligomycin-sensitive ATPase of rat liver mitochondria (Catterall and Pedersen, 1972). This observation is in agreement with the inability of F_1 preparations from rat liver (Catterall and Pedersen, 1972) and ox heart (Harris *et al.*, 1973) to bind AMP.

Specific inhibition of mitochondrial respiration and the partial reactions of oxidative phosphorylation by oligomycin and N,N'dicyclohexylcarbodiimide (Bulos and Racker, 1968; Lardy *et al.*, 1958) is dependent on an oligomycin-sensitivity-conferring protein (Beechey *et al.*, 1967). The insensitivity of the outer membrane ATPase to these inhibitors (Table 4.8) argues against such a protein subunit being associated with it. In addition, the outer membrane ATPase is insensitive to NaN_3 (Table 4.8), which is a direct specific inhibitor of the F_1-enzyme (Slater, 1955). These findings distinguish the outer membrane ATPase from the oligomycin-sensitive enzyme both in its native and F_1-forms. Further, the insensitivity of the outer membrane enzyme to thiol-specific reagents (Table 4.8) rules out the possibility that accessible thiol groups are involved at the active site.

Although the oligomycin-sensitive ATPase isolated from mitochondria catalyses the hydrolysis of ATP *in vitro*, it also functions in the direction of ATP synthesis if energy is provided in the form of a membrane potential or an ion-gradient (Skulachev, 1974; Moyle and Mitchell, 1975; Racker, 1972). There are a family of ATPases from the membranes of many organelles (e.g., nucleus, microsomes, sarcoplasmic reticulum, etc.) and plasma membranes which act as powerful ATP-hydrolyzing enzymes in the presence of activating metal ions (e.g., Mg^{2+}, Ca^{2+}, Na^{+}, K^{+}, etc.), but very little is known about their potential ATP-synthesizing ability *in vivo*. If the overall sum of the competing reactions involved in the formation of ATP by mitochondrial oxidative phosphorylation (200 nmoles of ATP formed/min per mg protein; Chao and Davis, 1972) and the hydrolysis of ATP by a variety of ATPases (microsomal, nuclear and sarcoplasmic reticulum) is considered, it is evident that more ATP would be hydrolyzed than formed, at least under the experimental conditions used *in vitro*. Accordingly, these reactions should result in a low steady-state

concentration of ATP/ADP in the cytoplasm. In contrast, in freeze-clamped spleen slices, for example, a rather high ATP/ADP ratio and energy charge ratio have been observed (Suter and Weidemann, 1976).

It was predicted on these grounds that the activity of such energy-dissipating ATPases must be under the control of regulator molecules (e.g., metabolic intermediates, end-products, etc.) or cytoplasmic protein-like components *in vivo*. Attempts were made in the present study to identify potential regulator molecules; none was found, however, that had the capability of regulating the ATPase activity to any measurable extent, either alone or in the presence of a cytoplasmic extract, (see Tables 4.3 to 4.6). These observations suggest that the cytoplasmic protein factor, if it exists, must be either: (a) very labile; or (b) detectable only when the cytoplasmic fraction is concentrated; or (c) expressed in the presence of some ligand molecule that was not tested. Inhibitor or regulator proteins have been identified and isolated only in the case of mitochondrial ATP-synthase (Pullman and Monroy, 1963; Horstman and Racker, 1970; Van de Stadt *et al.*, 1973; Fergusen *et al.*, 1977) and there are no reports of such proteins being associated with other membrane-bound ATPases in mammalian cells. The likelihood of there being inhibitors for individual ATPases that regulate their activity is not known.

4.5.3 Function of the ATPase in relation to outer membrane permeability

Spleen mitochondria resemble kidney-cortex and heart mitochondria in some respects, especially in their response to added bivalent metal ions. Addition of Mg^{2+} to all of these mitochondria prolongs state-3 respiration (Chance and Williams, 1956) and apparently uncouples them (Chao and Davis, 1972; Gmaj *et al.*, 1974; Vijayakumar and Weidemann, 1976) and also alters their Ca^{2+}-handling capacity (Gmaj *et al.*, 1974;

Sordahl, 1974; Jacobus *et al.*, 1975). It has been suggested, at least for rat kidney-cortex mitochondria, that a decrease observed in the transport of Ca^{2+} in the presence of Mg^{2+} may have been due to the involvement of the Mg^{2+}-stimulated outer membrane ATPase (Gmaj *et al.*, 1974). Unlike liver mitochondria, spleen, heart and kidney-cortex mitochondria exchange externally added [^{14}C] ADP with only a small proportion of their endogenous ademine nucleotide pool (see Chapter V). Finally, all of these mitochondria have a Mg^{2+}-stimulated ATPase associated with their outer membranes (Vijayakumar and Weidemann, 1976; Gmaj *et al.*, 1974). These observations suggest that there may be fundamental differences in the permeability properties of the outer membranes of different types of mitochondria, although it has been generally accepted that the liver mitochondrial outer membrane (for example) is freely permeable to low-molecular-weight substances (Ernster and Kuylenstierna, 1970). This hypothesis obviously needs more experimental support. Further work aimed at defining the role of the mitochondrial outer membrane ATPase in regulating the intracellular distribution of bivalent metal ions is discussed in the following chapter.

CHAPTER 5

POSSIBLE FUNCTIONAL ROLE OF THE OUTER MEMBRANE ATPase OF RAT SPLEEN MITOCHONDRIA

CHAPTER 5

POSSIBLE FUNCTIONAL ROLE OF THE OUTER MEMBRANE ATPase OF RAT SPLEEN MITOCHONDRIA

5.1 INTRODUCTION

It is apparent from the findings described in the previous Chapter that Cu^{2+} is the only agent tested that strongly influenced (inhibited) the activity of the ATPase from the outer membrane of rat spleen mitochondria. Based on this finding, Cu^{2+} has been used as a tool to inhibit the enzyme in an attempt to study its functional role. Since membrane-bound ATPases have been implicated in the transport of cations (Ca^{2+}, Mg^{2+}, Na^{+} and K^{+}) (see Chapter I for details), attention has been focussed on the possible role of this enzyme in the modulation of ion-transport by spleen mitochondria. Particular emphasis has been given to the transport of Ca^{2+} as the enzyme exhibits an absolute requirement for either Ca^{2+} or Mg^{2+} for activity and has a high affinity for Ca^{2+}.

A number of heavy metal ions such as Cu^{2+}, Hg^{2+}, Pb^{2+}, etc., have been shown to react with components of biological membranes (Passow *et al.*, 1961). Such interactions can displace the components that are normally bound at negatively charged sites and introduce positive charges. Thiol groups and sites that are normally uncharged are especially vulnerable to modification by heavy metals (Lehninger, 1962). When the interaction is extensive, the lipid-protein bilayer is disrupted and this leads to the loss of the permeability barrier across the membrane boundary. However, less extensive interactions have also been shown to produce alterations in the functions of membranes (Hwang *et al.*, 1972). In isolated mitochondria it has been shown that the addition of Pb^{2+} (Scott *et al.*, 1971; Cardona *et al.*, 1971), Zn^{2+} (Brierley *et al.*, 1968; Jacobus and Brierley, 1969) or Cu^{2+} (Varity and Gambell, 1968) produces changes in the passive permeability of the membranes to cations and anions, changes in energy-

dependent ion movements (see Parr and Harris, 1976) and changes in oxidative phosphorylation (Varity and Gambell, 1968). Cu^{2+} in particular has been shown to be one of the most effective activators of the energy-linked accumulation of cations by liver and heart mitochondria and is consequently capable of inducing high rates of swelling (Hwang *et al.*, 1972).

It was observed in the present study, that Ca^{2+}-induced swelling of rat liver mitochondria was enhanced by the addition of Cu^{2+}, whereas, under identical conditions, the swelling of spleen mitochondria was inhibited markedly. Further experiments were performed to study the effects of Cu^{2+} and Mg^{2+} on the $^{45}Ca^{2+}$-handling capacity of spleen mitochondria. These experiments were designed to test the hypothesis that the outer membranes may be less permeable than those of liver to bivalent metal ions (see Chapter IV; Vijayakumar and Weidemann, 1977). Furthermore, the influence of the intact outer membrane and the effect of its removal on the translocation of adenine nucleotides and the modulation of this process by bivalent cations was tested with a view to gaining insight into the general mechanism of metabolite transport by spleen mitochondria.

5.2 MATERIALS

^{45}Ca, [8-^{14}C]-ATP, and [8-^{14}C]-ADP were purchased from The Radiochemical Centre, Amersham, U.K. Analytically pure $CaCl_2$, atractyloside and bovine serum albumin were obtained from the sources described in previous Chapters. All other chemicals used were of the highest purity available commercially. Millipore filters (0.5μ) were from Gelman Instrument Co., Ann Arbor, MI., U.S.A.

5.3 METHODS

5.3.1 Isolation of rat spleen mitochondria, mitoplasts and outer membranes

Mitochondria were isolated from rat spleen and liver as described before (Chapter II and III). The mitoplasts and the outer membranes were prepared from the isolated intact rat spleen mitochondria by digitonin fractionation as described before (Chapter III). Mitoplasts from rat liver mitochondria were prepared as described by Schnaitmann *et al.* (1967).

5.3.2 Measurement of mitochondrial swelling

Mitochondria from rat liver (6mg of protein) or spleen (3mg of protein) were suspended in an incubation medium (2.5ml final volume) consisting of sucrose (250mM), Hepes buffer (10mM; pH 7.4) and ATP (2mM) or succinate (2mM). When Cu^{2+} was included in the incubation medium, it was added at the concentrations indicated in the Legends. Swelling was induced by addition of Ca^{2+} and was followed, using a Zeiss spectrophotometer attached to a Beckman recorder, by the fall in A_{520}.

5.3.3 Measurement of time-dependent Ca^{2+}-transport in rat spleen mitochondria

Rat spleen mitochondria (1.5mg of protein) or mitoplasts (1.0mg of protein) were incubated for 1.0min at 10°C in a reaction chamber containing sucrose (250mM), Hepes buffer (10mM; pH 7.4) and ATP (1.0mM) or succinate (2.0mM) in a final volume of 1.0ml. Ca^{2+} uptake was initiated by adding ^{45}Ca (2500 d.p.m./nmol) to give a final concentration of 100µM. Samples (100µl) were removed at 10s intervals for 60s and placed in Eppendorf centrifuge tubes containing a Ruthenium red-EGTA mixture to stop Ca^{2+}-uptake (Reed and Bygrave, 1974). The tubes were centrifuged immediately in an Eppendorf centrifuge for 2 min and, after aspiration of the supernatants, the pellets were dissolved in concentrated formic

acid (100µl). Control experiments were carried out under identical conditions except that ATP and succinate were not included in the incubation medium. The contents were transferred to scintillation vials quantitatively and the ^{45}Ca was counted as described in Chapter II.

5.3.4 Measurement of Ca^{2+} uptake by rat spleen mitochondria at fixed time intervals

Ca^{2+} uptake by rat spleen mitochondria was monitored essentially as described above (5.3.3) except that it differed in the following ways: (a) mitochondria (0.15mg of protein) were incubated at 10°C in Eppendorf centrifuge tubes containing the reaction medium described above in a final volume of 0.1ml; (b) Mg^{2+} and/or Cu^{2+} were present in the medium at the concentrations indicated in the Figures; and (c) ^{45}Ca uptake was stopped at the end of 20s by addition of a Ruthenium red-EGTA mixture.

5.3.5 Measurement of Ca^{2+} uptake by outer membrane vesicles prepared from spleen mitochondria

Outer membrane vesicles (0.4mg of protein) prepared from rat spleen mitochondria were incubated for 30s at 30°C in a reaction medium containing sucrose (250mM), Hepes (10mM; pH 7.4), MgCl (2.0mM), ATP (0.5mM) and P_i-buffer (2 mM; pH 7.4) in a final volume of 1.0ml. Ca^{2+} uptake was initiated by adding sufficient ^{45}Ca (100,000 d.p.m./nmol) to give a final concentration of 50µM. Samples (100µl) were removed at 0, 0.5, 1, 2, 5 and 10 min intervals and were filtered rapidly (<5sec) through a Millipore filter (0.45µ) under negative pressure. The adherent vesicles were washed immediately with a solution (10ml) containing sucrose (250mM), Hepes (5mM; pH 7.4) and $MgSO_4$ (5mM) that the Millipore filter papers had been soaked in previously to minimize the non-specific binding of ^{45}Ca. The Millipore filters were dried, placed in scintillation vials and the ^{45}Ca radioactivity determined as described in Chapter II.

5.3.6 Measurement of adenine nucleotide exchange

Exchange of adenine nucleotides by rat spleen mitochondria was monitored by the "forward exchange" method of Pfaff and Klingenberg (1968). Rat spleen mitochondria (1.0mg of protein) or mitoplasts (1.0mg of protein) were incubated in Eppendorf centrifuge tubes for 1.0 min at 0°C in a reaction medium containing sucrose (250mM), Tris-maleate buffer (10mM; pH 7.0) and EDTA-Tris (2.0mM; pH 7.0) in a final volume of 1.0ml. The reactions were initiated by addition of appropriate amounts of labelled nucleotides, as indicated in the Legends, and were terminated at definite time intervals by addition of atractyloside (50μM). [The additions were made on "plumpers" (stirrers) set in a holder so that four separate reactions could be initiated or terminated simultaneously.] The tubes were centrifuged immediately in an Eppendorf centrifuge for 2½ min. After a small aliquot (200μl) of the clear supernatant was removed for estimation of the radioactive adenine nucleotides, the remainder was removed by aspiration. The pellet was washed with sucrose (1.0ml; 3.0M) and was dissolved in concentrated formic acid (200μl). Control experiments were carried out under identical conditions except that atractyloside (50μM) was added to the incubation medium prior to initiation of the reaction.

Percentage exchange of the endogenous adenine nucleotides was calculated by the method of Pfaff and Klingenberg (1968):

i.e.,

$$\text{Percentage exchange} = \frac{\text{specific activity of ATP \& ADP (in)}}{\text{specific activity of ATP \& ADP (out)}} \times 100$$

$$\text{where specific activity (in)} = \frac{\text{d.p.m. in the pellet}}{\text{nmols of ATP \& ADP in the pellet}}$$

$$\text{specific activity (out)} = \frac{\text{total d.p.m. added - d.p.m. in the pellet}}{\text{nmols of ATP \& ADP added}}$$

Fig.5.1 Induction and inhibition of Ca^{2+}-stimulated mitochondrial swelling by Cu^{2+}

Mitochondrial swelling was conducted in a reaction medium described in the Experimental section. Ca^{2+} was added to initiate swelling at the concentrations indicated and was monitored as described in the text. Where indicated, Cu^{2+} was added to the reaction medium to give the final concentrations indicated.

Fig (a) - rat liver mitochondria

Fig (b) - rat spleen mitochondria

Fig (a)

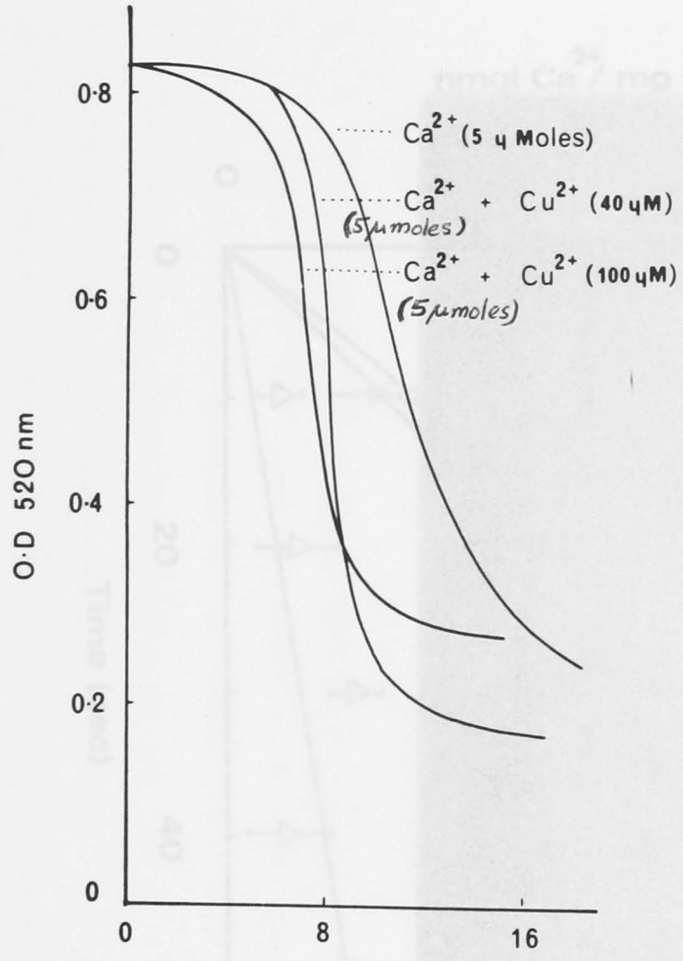
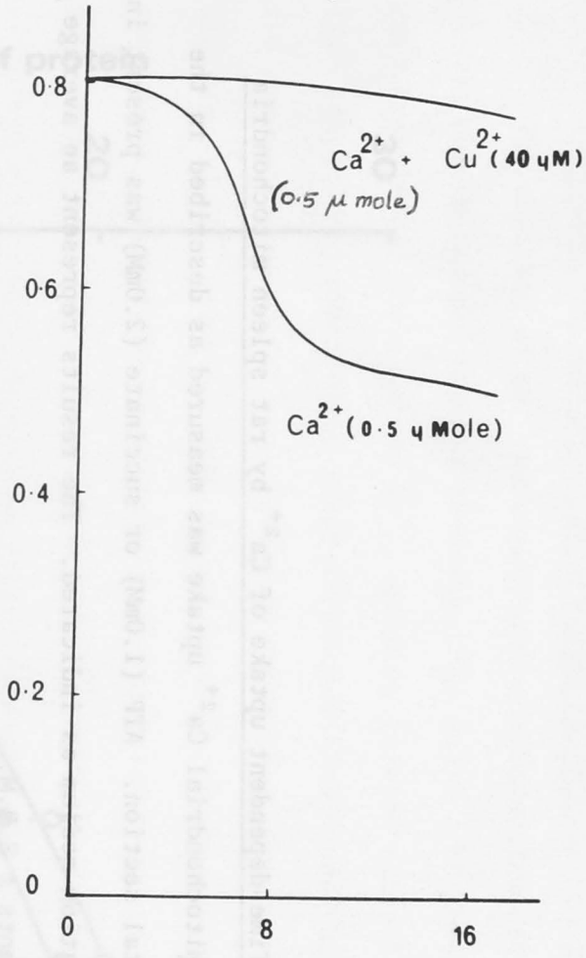


Fig (b)

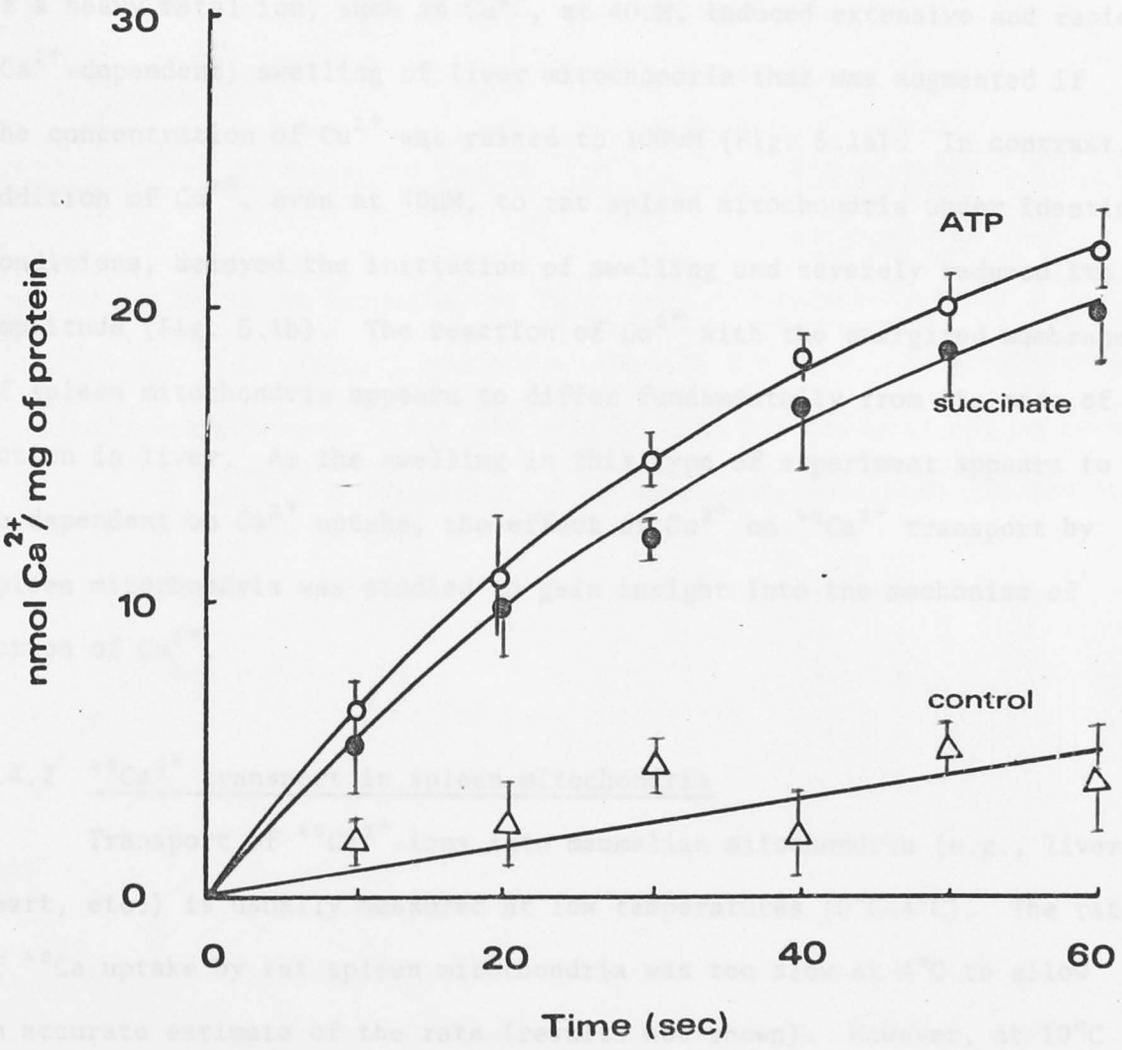


Time (Min)

Fig.5.2 Time-dependent uptake of Ca^{2+} by rat spleen mitochondria

Mitochondrial Ca^{2+} uptake was measured as described in the Experimental section. ATP (1.0mM) or succinate (2.0mM) was present in the incubation medium as indicated. The results represent an average of 3 experiments ± S.E.M.

Control experiment is done without any added substrates.


30
20
10
0
nmol Ca^{2+}/ mg of protein
ATP
succinate
control
0
20
40
60
Time (sec)

5.3.7 Estimation of adenine nucleotides

The adenine nucleotides were estimated using the coupled-enzyme methods described in Chapter IV.

5.4 RESULTS

5.4.1 Induction and inhibition of swelling by Cu^{2+}

The addition of Ca^{2+} to mitochondria isolated from either rat liver or rat spleen induced passive swelling (Fig. 5.1). The further addition of a heavy metal ion, such as Cu^{2+}, at 40μM, induced extensive and rapid (Ca^{2+}-dependent) swelling of liver mitochondria that was augmented if the concentration of Cu^{2+} was raised to 100μM (Fig. 5.1a). In contrast, addition of Cu^{2+}, even at 40μM, to rat spleen mitochondria under identical conditions, delayed the initiation of swelling and severely reduced its amplitude (Fig. 5.1b). The reaction of Cu^{2+} with the energized membranes of spleen mitochondria appears to differ fundamentally from its mode of action in liver. As the swelling in this type of experiment appears to be dependent on Ca^{2+} uptake, the effect of Cu^{2+} on $^{45}Ca^{2+}$ transport by spleen mitochondria was studied to gain insight into the mechanism of action of Cu^{2+}.

5.4.2 $^{45}Ca^{2+}$ transport in spleen mitochondria

Transport of $^{45}Ca^{2+}$-ions into mammalian mitochondria (e.g., liver, heart, etc.) is usually measured at low temperatures (0°C-4°C). The rate of ^{45}Ca uptake by rat spleen mitochondria was too slow at 4°C to allow an accurate estimate of the rate (results not shown). However, at 10°C there was a significant rate of ^{45}Ca uptake that was linear for about 40s when either ATP (1.0mM) or succinate (2.0mM) was used as substrate (Fig. 5.2). In the absence of an added substrate to energize the membranes, or when Ruthenium red was included in the incubation medium, the rate of Ca^{2+} uptake was significantly lowered. These findings support

Fig.5.3 Modulation by Cu^{2+} and Mg^{2+} of the transport of Ca^{2+} by rat spleen mitochondria

Mitochondrial Ca^{2+} transport was measured as described in the Experimental section. ATP (1.0mM), succinate (2.0mM), Cu^{2+} and Mg^{2+} were added to the incubation medium to give the final concentrations indicated. The results represent an average of 3 experiments ± S.E.M.

Fig (a)

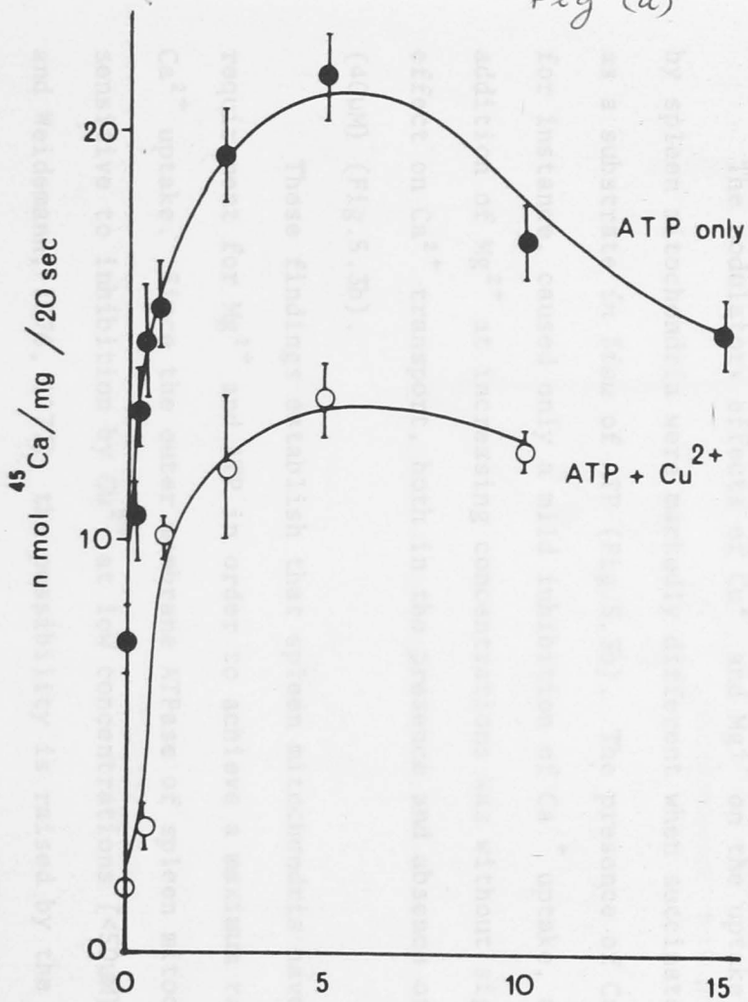
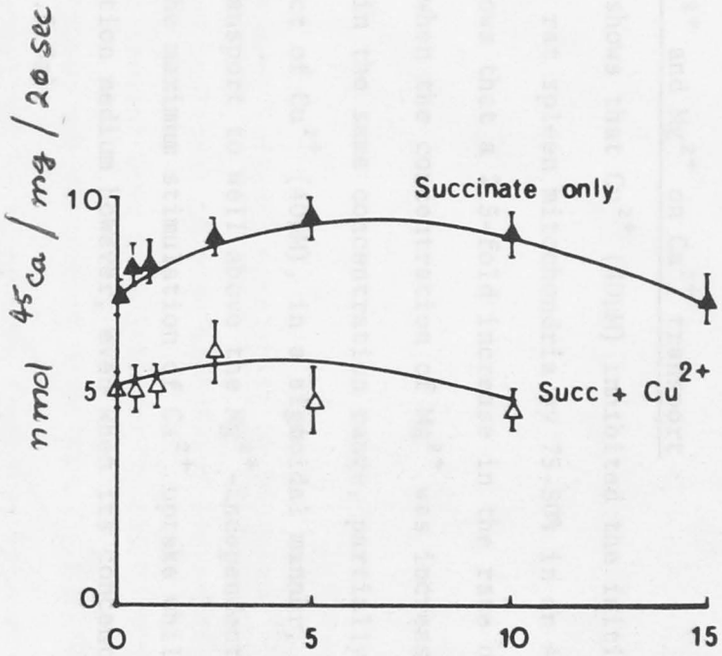


Fig (b)



Conc of Mg^{2+} ions (mM)

the view that rat spleen mitochondria have the conventional Ruthenium-red-sensitive, energy-dependent Ca^{2+} transporting system similar to that present in the mitochondria of other tissues.

5.4.3 Effect of Cu^{2+} and Mg^{2+} on Ca^{2+} transport

Fig. (5.3a) shows that Cu^{2+} (40μM) inhibited the initial rate of Ca^{2+} transport into rat spleen mitochondria by 75-80% in an ATP-supported system. It also shows that a 2.5-fold increase in the rate of Ca^{2+}-transport occurred when the concentration of Mg^{2+} was increased (Fig. 5.3a). Mg^{2+}, furthermore, in the same concentration range, partially relieved the inhibitory effect of Cu^{2+} (40μM), in a sigmoidal manner, and restored the rate of Ca^{2+} transport to well above the Mg^{2+}-independent rate. Mg^{2+} failed to restore the maximum stimulation of Ca^{2+} uptake while Cu^{2+} was present in the reaction medium however, even when its concentration was raised to 10mM (Fig.5.3a).

The modulatory effects of Cu^{2+} and Mg^{2+} on the uptake of Ca^{2+} by spleen mitochondria were markedly different when succinate was used as a substrate *in lieu* of ATP (Fig.5.3b). The presence of Cu^{2+} (40μM), for instance, caused only a mild inhibition of Ca^{+} uptake, and the addition of Mg^{2+} at increasing concentrations was without significant effect on Ca^{2+} transport, both in the presence and absence of Cu^{2+} (40μM) (Fig.5.3b).

These findings establish that spleen mitochondria have an absolute requirement for Mg^{2+} and ATP in order to achieve a maximum rate of Ca^{2+} uptake. Since the outer membrane ATPase of spleen mitochondria is sensitive to inhibition by Cu^{2+} at low concentrations (<50μM) (Vijayakumar and Weidemann, 1976, 1977), the possibility is raised by the present study that this enzyme may catalyze an ATP-dependent uptake of Ca^{2+} ions across the outer mitochondrial membrane (stage I) to the site of the Ruthenium red-sensitive process at the inner membrane (stage II) (see Scheme I).

Fig.5.4 Ca^{2+} transport in mitoplasts prepared from rat spleen mitochondria

Experimental conditions were as described before (Fig.5.2), except that mitoplasts (1.0mg of protein) were used instead of mitochondria. ATP (1.0mM) and/or Mg^{2+} (5.0mM) were present in the reaction medium where indicated. The results represent an average of duplicate experiments from two different mitoplast preparations.

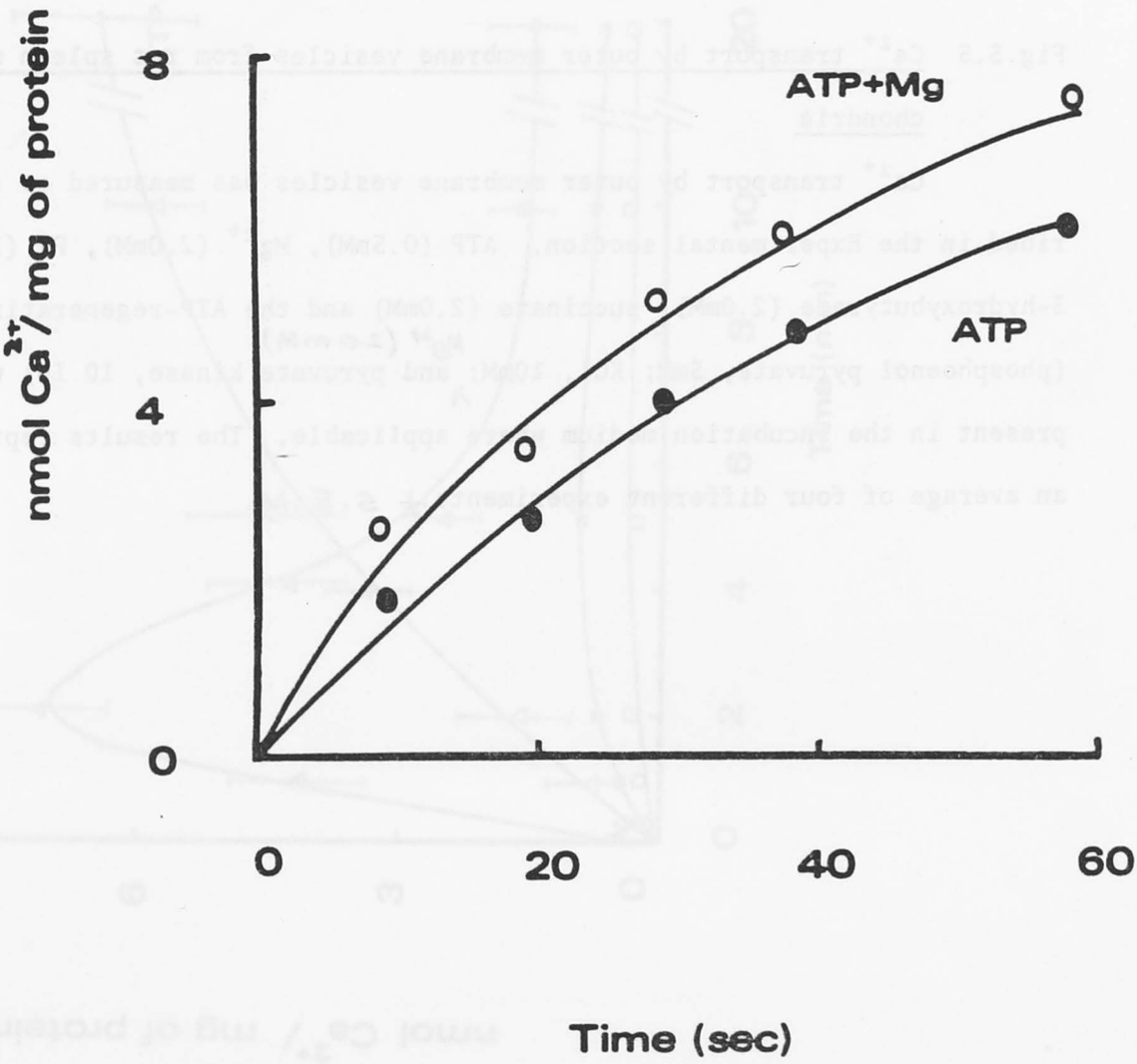

nmol Ca^{2+} / mg of protein
8
4
0
ATP+Mg
ATP
0
20
40
60
Time (sec)

Fig.5.5 Ca^{2+} transport by outer membrane vesicles from rat spleen mitochondria

Ca^{2+} transport by outer membrane vesicles was measured as described in the Experimental section. ATP (0.5mM), Mg^{2+} (2.0mM), Pi (2.0mM), 3-hydroxybutyrate (2.0mM), succinate (2.0mM) and the ATP-regenerating system (phosphoenol pyruvate, 5mM; KCl, 10mM; Mg^{2+} (2.0 mM) and pyruvate kinase, 10 IU) were present in the incubation medium where applicable. The results represent an average of four different experiments ± S.E.M.

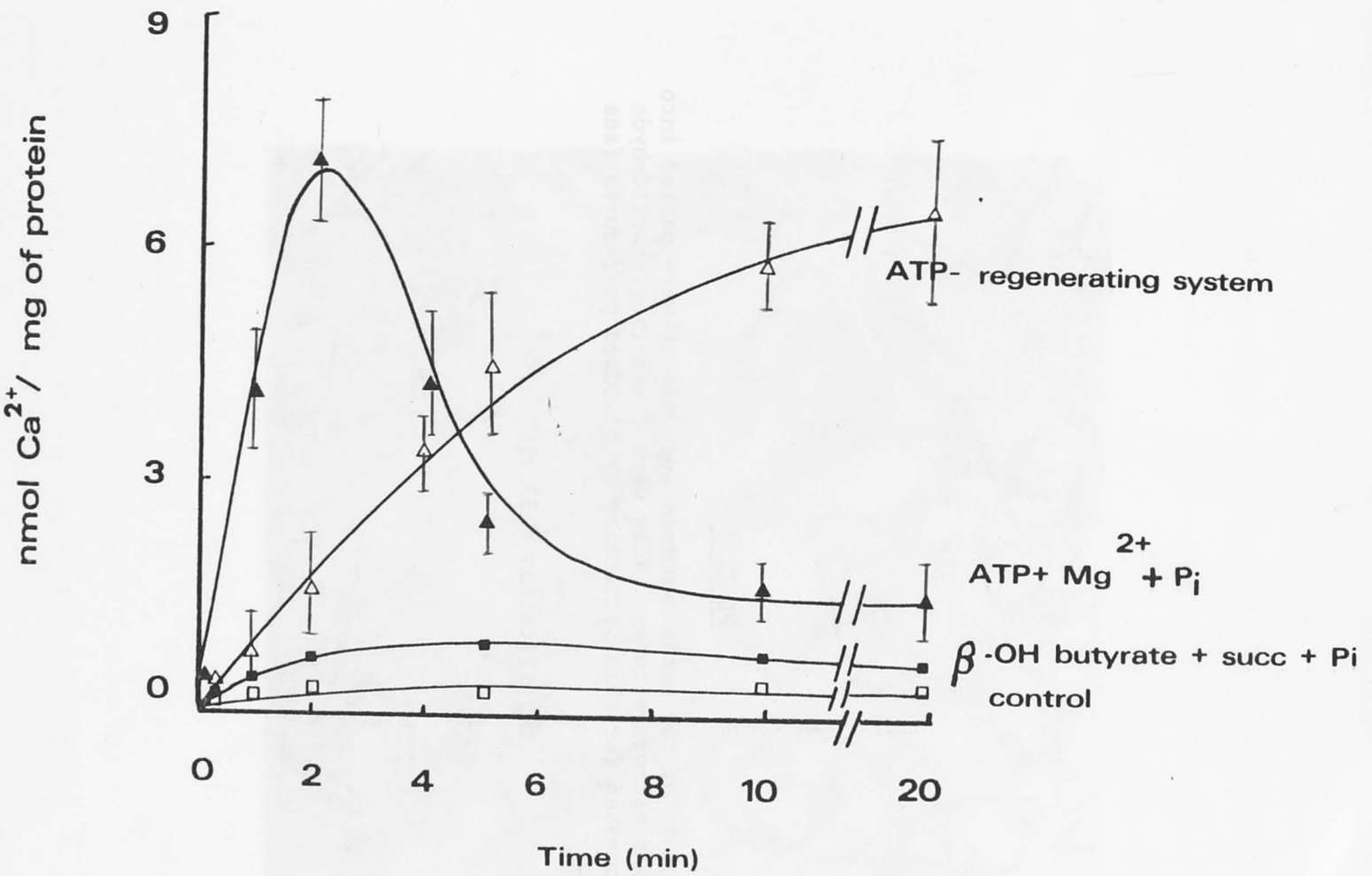
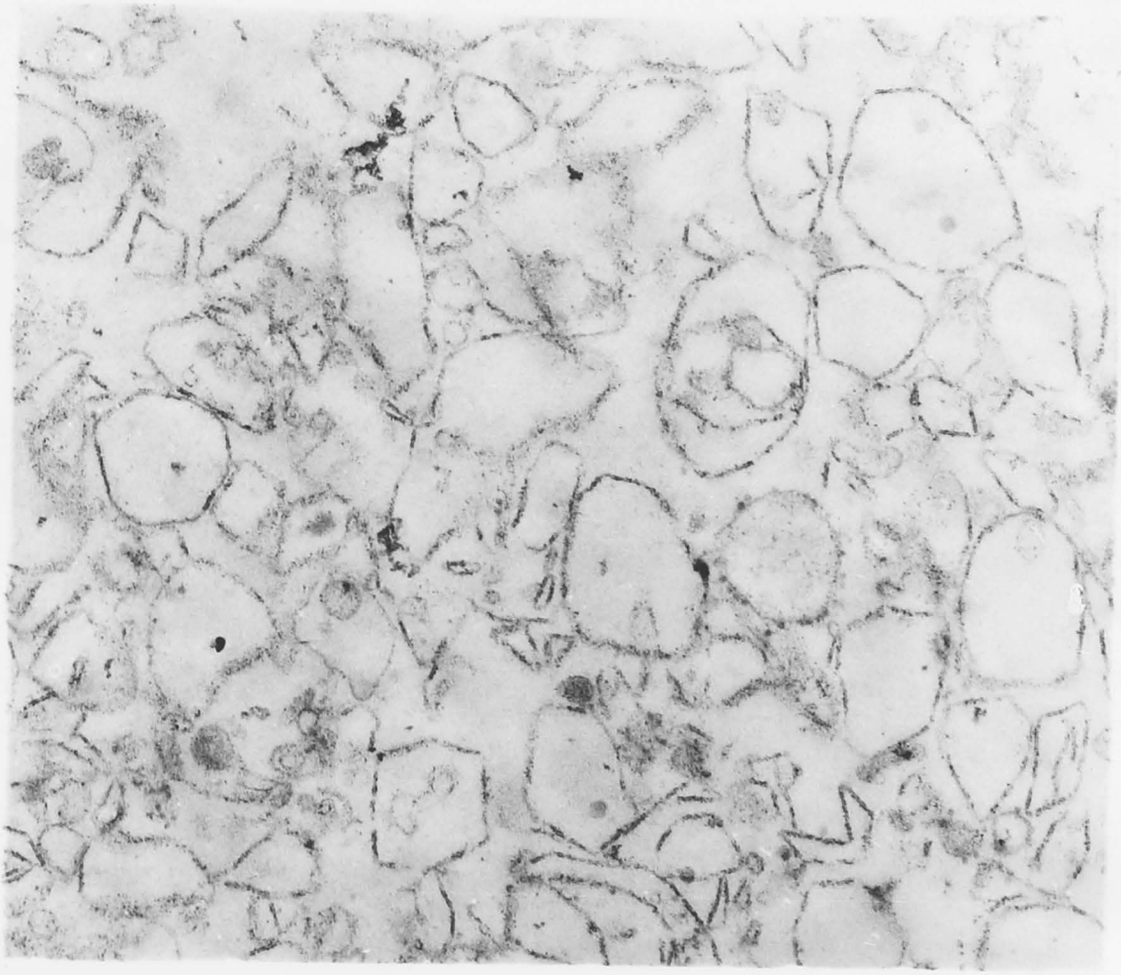

9
6
3
0
nmol Ca^{2+}/ mg of protein
0
2
4
6
8
10
20
Time (min)
ATP- regenerating system
ATP+ Mg^{2+} + P_i
β-OH butyrate + succ + Pi
control

PLATE 1

Small amounts of the outer membrane suspensions were packed into pellets in Beckman microfuge tubes. They were fixed in glutaraldehyde and OsO_4 and processed for thin-sectioning by standard procedures (see Glauert, 1975).

Magnification × 33,000



5.4.4 Transport of Ca^{2+} by mitoplasts prepared from rat spleen mitochondria

If there is an energy barrier for the movement of Ca^{2+} ions across the outer membranes of rat spleen mitochondria then, according to this hypothesis, removal of the outer membranes should: (a) stimulate the maximum rate of uptake of Ca^{2+} ions quite independently of added Mg^{2+}; and (b) abolish the Mg^{2+}-dependent stimulation of Ca^{2+} uptake in the ATP-supported system. Contrary to such predictions, spleen mitochondria exhibited a relatively low rate of uptake of Ca^{2+} upon removal of outer membranes, although, as predicted by the hypothesis (see b), this process was not significantly stimulated by added Mg^{2+} (Fig.5.4).

5.4.5 Ca^{2+} uptake by outer membrane vesicles of rat spleen mitochondria

Freshly prepared outer membranes from rat spleen mitochondria formed tightly-sealed vesicles in a manner similar to that observed with rat liver mitochondria (see Schnaitman *et al.*, 1967; see Plate 1). When the vesicles were suspended in a buffered sucrose medium containing Mg^{2+} and ATP they were capable of accumulating $^{45}Ca^{2+}$ for about 2 min (Fig.5.5). The accumulated ^{45}Ca leaked out of the vesicles rapidly on the exhaustion of ATP despite the presence of P_i. However, when the incubation medium was supplemented with an ATP-regenerating system (i.e., phosphoenolpyruvate, 5.0mM; KCl, 10mM; and pyruvate kinase, 10 I.U.) the vesicles accumulated Ca^{2+} linearly for about 2½ min and retained it for more than 20 min. No measurable Ca^{2+} uptake was observed in the absence of either Mg^{2+}, ATP or P_i. Similarly, when 3-hydroxybutyrate, succinate and P_i were used to support Ca^{2+} transport, as described for heart mitochondrial inner membrane vesicles (Loyter *et al.*, 1969), no significant Ca^{2+} accumulation was detected. These findings support the view that there may be an energy-dependent Ca^{2+} accumulation across the outer membranes of spleen mitochondria that preceeds and is distinct from Ca^{2+}

Fig.5.6 Adenine nucleotide exchange by mitochondria from liver and spleen

Adenine nucleotide exchange by mitochondria was monitored at 0°C as described in the Experimental section. The reactions were initiated by adding [^{14}C]-ADP (2μCi/μmole) to give a final concentration of 10μM in the reaction mixture. The reactions were terminated at different time intervals by addition of atractyloside (50μM). The percentage exchange of nucleotides was calculated as described in the text.

(o) rat liver mitochondria

(▲) rat spleen mitochondria

(●) rat spleen mitoplasts

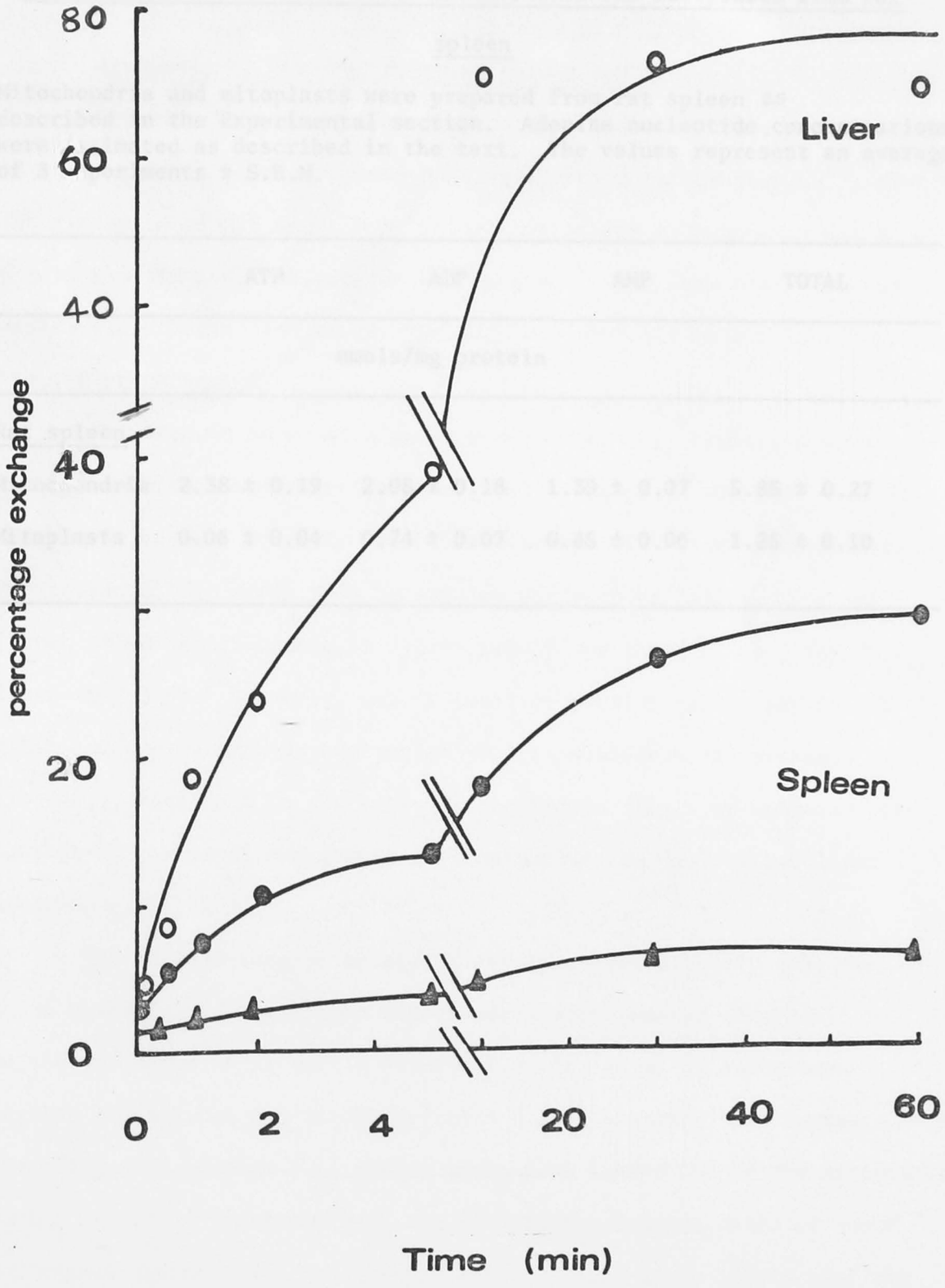

80
60
40
40
20
0
percentage exchange
Liver
Spleen
0
2
4
20
40
60
Time (min)

Table 5.1

Adenine nucleotide contents of mitochondria and mitoplasts from rat spleen

Mitochondria and mitoplasts were prepared from rat spleen as described in the Experimental section. Adenine nucleotide concentrations were estimated as described in the text. The values represent an average of 3 experiments ± S.E.M.

	ATP	ADP	AMP	TOTAL
	nmols/mg protein			
Rat spleen				
Mitochondria	2.38 ± 0.19	2.08 ± 0.18	1.39 ± 0.07	5.85 ± 0.27
Mitoplasts	0.06 ± 0.04	0.74 ± 0.07	0.45 ± 0.06	1.25 ± 0.10

accumulation across the inner membrane.

5.4.6 Characteristics of the translocation of adenine nucleotides by mitochondria isolated from rat spleen and liver

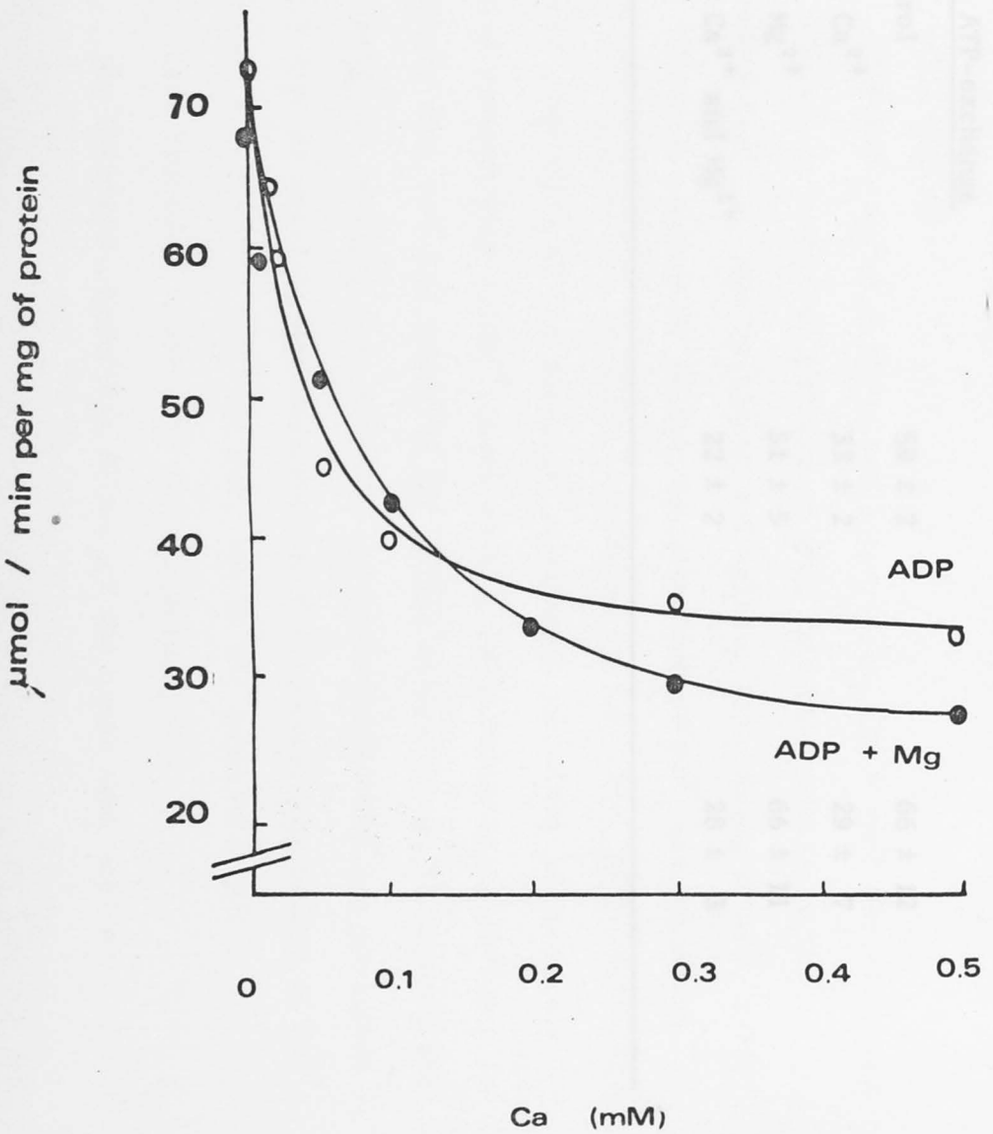
In order to test the possibility that there may be a permeability barrier across the outer membrane to metabolites other than Ca^{2+}, the translocation of ATP and ADP by intact rat spleen mitochondria and mitoplasts was studied. Effects of Ca^{2+} and Mg^{2+} ions were tested in this system also to gain additional insight into possible relationships between the two processes.

The translocation of adenine nucleotides into tightly-coupled rat spleen mitochondria (respiratory control ratio of not less than 5) differs in several respects from that observed with liver mitochondria. For instance, the total pool of adenine nucleotides (ATP + ADP + AMP) in rat spleen mitochondria is approximately one third of that found in liver (Fig.5.6). Secondly, only a small proportion (about 6%) of the total endogenous adenine nucleotide pool is available for exchange with externally added ADP in rat spleen mitochondria (Fig.5.6) compared with 60-100% of the total endogenous adenine nucleotide pool in rat liver mitochondria (Fig.5.6).

The initial rate of uptake of ADP increased slightly when the outer membranes of rat spleen mitochondria were removed (Fig.5.6); but, as a consequence, a five-fold reduction in the level of endogenous adenine nucleotides was observed (Table 5.1). Although this suggests that the endogenous adenine nucleotides might have leaked out of the mitoplasts during digitonin fractionation, the respiratory control ratio of about 2.0 argues against such an interpretation. Thus, it is likely that the majority of the adenine nucleotides may be located outside the inner membrane boundary, although there is no conclusive additional evidence to support this.

Fig.5.7 Modulation by Ca^{2+} and Mg^{2+} of the translocation of adenine nucleotides by rat spleen mitochondria

Adenine nucleotide exchange was monitored as described in the Experimental section. The reaction medium contained Mg^{2+} (4.0mM) or Ca^{2+}, where appropriate, at the concentrations indicated. The reactions were initiated by addition of [^{14}C]-ADP (8μCi/μmole) or [^{14}C]-ATP (8μCi/μmol) to give a final concentration of 100μM in the medium.


µmol / min per mg of protein
70
60
50
40
30
20
0
0.1
0.2
0.3
0.4
0.5
ADP
ADP + Mg
Ca (mM)

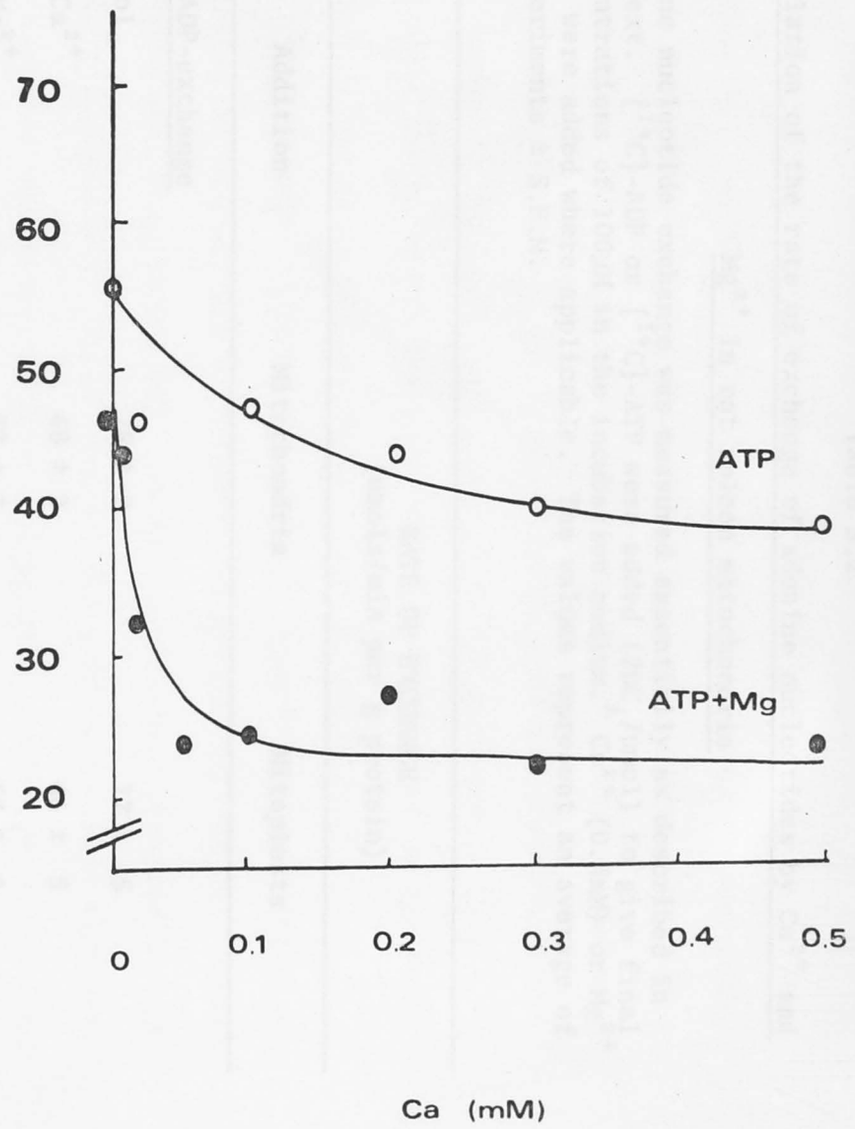

70
60
50
40
30
20
0
0.1
0.2
0.3
0.4
0.5
ATP
ATP+Mg
Ca (mM)

Table 5.2

Modulation of the rate of exchange of adenine nucleotides by Ca^{2+} and Mg^{2+} in rat spleen mitochondria

Adenine nucleotide exchange was measured essentially as described in the text. [^{14}C]-ADP or [^{14}C]-ATP were added (2μC./μmol) to give final concentrations of 100μM in the incubation medium.^{i} Ca^{2+} (0.4mM) or Mg^{2+} (4mM) were added where applicable. The values represent an average of 3 Experiments ± S.E.M.

	RATE OF EXCHANGE (nmols/min per g protein)	
Addition	Mitochondria	Mitoplasts
(a) ADP-exchange		
Control	78 ± 2	77 ± 5
Plus Ca^{2+}	48 ± 2	46 ± 5
Plus Mg^{2+}	77 ± 3	63 ± 5
Plus Ca^{2+} and Mg^{2+}	25 ± 1	25 ± 1
(b) ATP-exchange		
Control	59 ± 2	66 ± 12
Plus Ca^{2+}	33 ± 2	29 ± 7
Plus Mg^{2+}	51 ± 5	66 ± 11
Plus Ca^{2+} and Mg^{2+}	22 ± 2	28 ± 3

Fig.5.7 shows the influence of added Ca^{2+} and Mg^{2+} on the rate of adenine nucleotide translocation in rat spleen mitochondria. ADP translocation was inhibited by about 50-60% in response to increasing Ca^{2+} in the medium and this was not influenced significantly by added Mg^{2+} (4.0mM). Ca^{2+} added in the same concentration range inhibited the rate of translocation of ATP by only 20-25%; however, addition of Mg^{2+} (4mM) augmented the inhibitory effect of Ca^{2+}dramatically. This observation may be due to the conversion of ATP into ADP by the outer membrane ATPase which depends on Mg^{2+} for its activity.

The influence of added Ca^{2+} and Mg^{2+} ions on the translocation of adenine nucleotides was also studied in mitoplasts from rat spleen mitochondria. Since essentially similar results were obtained in both systems (see Table 5.2), these observations seem to indicate that the outer membrane ATPase may not have a significant influence on the translocation of adenine nucleotides.

5.5 DISCUSSION

The addition of a heavy metal ion (e.g., Cu^{2+}) to liver mitochondria induced, at low concentration (40μM), rapid and extensive Ca^{2+}-dependent swelling (see Cederbaum and Wainio, 1970; Hwang *et al.*, 1972). This effect was augmented if the concentration of Cu^{2+} was raised to 100μM (Fig.5.1). Cu^{2+} may have interacted, in this case, with negatively-charged components of the energized membranes, resulting in increased passive permeability to Ca^{2+} (Azzi *et al.*, 1971; Hwang *et al.*,1972). In contrast, when Cu^{2+} was added to rat spleen mitochondria, it delayed the initiation of swelling even at 40 μM, and severely reduced its amplitude (Fig.5.1). As swelling was dependent on Ca^{2+}-uptake, the effect of Cu^{2+} on spleen mitochondria may have been due to the inhibition of:

(a) Ca^{2+} transport across the inner membrane; or

(b) the oligomycin-sensitive ATPase of the inner membrane; or

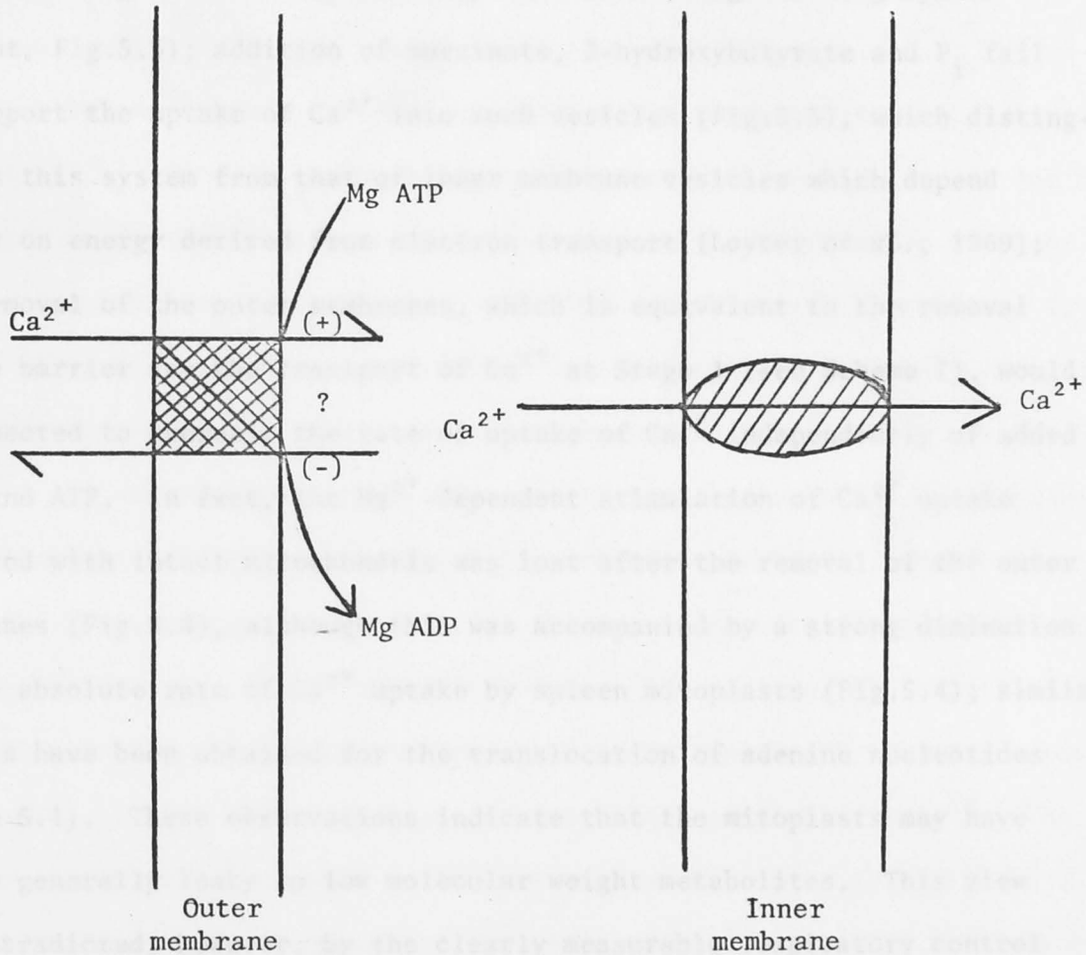
(c) the oligomycin-insensitive, Ca^{2+} and Mg^{2+}-stimulated ATPase of the outer membrane; or

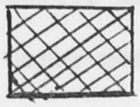
(d) some other step.

In the first place, since Cu^{2+} is only a mild inhibitor of succinate-supported Ca^{2+} transport (Fig.5.3b), the transport of Ca^{2+}-ions across the inner membranes of spleen mitochondria is practically unaffected by Cu^{2+}. Secondly, Cu^{2+} at low concentrations (e.g., 5-7nmols/mg of protein) stimulates the oligomycin-sensitive ATPase of the mitochondrial inner membranes (Hwang *et al.*, 1972); whilst this observation is consistent with the stimulation of (ATP-supported) Ca^{2+}-induced swelling by Cu^{2+}, in liver mitochondria, it does not explain the inhibitory effects noted with those from spleen. If the action of Cu^{2+} is due to the inhibition of the oligomycin-insensitive outer membrane ATPase (Vijayakumar and Weidemann, 1977) this strengthens the case for the enzyme playing a role in Ca^{2+}-transport by catalyzing the uptake of Ca^{2+} into the inter-membrane space at the expense of ATP hydrolysis. This being so, the outer membranes of spleen mitochondria should have selective permeability properties quite unlike those of liver.

In the present study, transport of Ca^{2+} into spleen mitochondria supported by ATP was stimulated up to 2.5-fold when the Mg^{2+} concentration was increased (Fig.5.3a). In contrast, when the Ca^{2+} uptake was supported by succinate it was comparatively unaffected by Mg^{2+} (Fig.5.3b). These observations suggest that the addition of Mg^{2+} and ATP (but not succinate) stimulates the outer membrane ATPase (Vijayakumar and Weidemann, 1976) to provide energy necessary to overcome an additional barrier to the transport of Ca^{2+}. This view is strengthened by the observation that, whereas Mg^{2+} stimulates the uptake of Ca^{2+} by spleen mitochondria, it has been reported either to have no effect [e.g., in heart mitochondria (Scarpa and Graziotti, 1973)] or to compete with the transport of Ca^{2+} in various mitochondria [e.g., in mitochondria isolated from liver (Vainio

Scheme I





outer membrane ATPase

Ca^{2+}-transporter

et al., 1970); from heart (Sordahl, 1974; Carafoli *et al.*, 1975); and from kidney-cortex (Gmaj *et al.*, 1974)].

Additional evidence that supports the selective permeability of the outer membranes of spleen mitochondria can be summarized as follows: (a) tightly-sealed vesicles of these membranes transport Ca^{2+} in the presence of Mg^{2+} and ATP (preferably with an ATP-regenerating system present, Fig.5.5); addition of succinate, 3-hydroxybutyrate and P_i fail to support the uptake of Ca^{2+} into such vesicles (Fig.5.5), which distinguishes this system from that of inner membrane vesicles which depend solely on energy derived from electron transport (Loyter *et al.*, 1969); (b) removal of the outer membranes, which is equivalent to the removal of the barrier for the transport of Ca^{2+} at Stage 1 (see Scheme I), would be expected to increase the rate of uptake of Ca^{2+} independently of added Mg^{2+} and ATP. In fact, the Mg^{2+}-dependent stimulation of Ca^{2+} uptake observed with intact mitochondria was lost after the removal of the outer membranes (Fig.5.4), although this was accompanied by a strong diminution in the absolute rate of Ca^{2+} uptake by spleen mitoplasts (Fig.5.4); similar results have been obtained for the translocation of adenine nucleotides (Table 5.1). These observations indicate that the mitoplasts may have become generally leaky to low molecular weight metabolites. This view is contradicted, however, by the clearly measurable respiratory control ratio of 2-3 exhibited by the mitoplasts. If the outer membranes *are* impermeable to the metabolites suggested by the hypothesis, there could be a distinct metabolite pool present in the inter-membrane space. The removal of the outer membranes would thus greatly diminish the total metabolite pool size of mitoplasts compared with that of whole mitochondria. This is in fact seen in the case of the mitochondrial adenine nucleotides (Table 5.1).

Translocation of adenine nucleotides is inhibited by atractyloside and that of Ca^{2+} by Ruthenium red in spleen mitochondria. If this is

the case, the inhibitors must have direct access to the carrier molecules located at the inner membrane (Chapter I, Table 1.4) which argues that the outer membrane may be selectively permeable to low-molecular-weight substances. Most of the evidence considered in this Chapter supports the hypothesis that the outer membrane may have selective permeability properties and suggests that there may be a distinct metabolite pool present in the inter-membrane space.

Taken together, these findings support the proposal that transport of Ca^{2+} into spleen mitochondria may occur in two stages, as indicated in Scheme I: (a) transport of Ca^{2+} across the outer membrane into the inter-membrane space catalyzed by the Mg^{2+}-stimulated ATPase (Stage I); and (b) subsequent transport of Ca^{2+} across the inner membrane catalyzed by the Ruthenium red-sensitive Ca^{2+}-transporter (Stage II).

In accordance with the "two stage" hypothesis, addition of either succinate or ATP alone should not support even the basal rate of Ca^{2+} uptake into spleen mitochondria, as this would require ATP plus Mg^{2+} to overcome the barrier at Stage I. However, succinate or ATP supported the basal rate of Ca^{2+} uptake independently of Mg^{2+} plus ATP (Fig.5.2) which suggests that the outer membrane is relatively permeable to Ca^{2+}. The stimulation of Ca^{2+} uptake in the presence of Mg^{2+} plus ATP (Fig.5.3) could be due to the reversal of the efflux of Ca^{2+} (from the inter-membrane space to the extramitochondrial space) catalyzed by the outer membrane ATPase. The inability of succinate plus Mg^{2+} to increase the uptake of Ca^{2+} is consistent with the above view, as Mg^{2+}-ATP but not Mg^{2+}-succinate is the effective substrate for the outer membrane ATPase.

CHAPTER 6

GENERAL DISCUSSION

CHAPTER 6 .

GENERAL DISCUSSION

6.1 ROLE OF GLUCOSE AS A RESPIRATORY FUEL IN LYMPHOID TISSUES

Rat thymus lymphocytes, which have a very low glycogen content (Puckle, 1974; Nordeen and Young, 1977), depend to a large extent on exogenous glucose and endogenous triglycerides for maintenance of their energy supply. Similarly, rat spleen tissue, which contains mainly lymphocytes (90%) and a small percentage of macrophages (1.5%) and polymorphonuclear leukocytes (5.2%) (see Suter, 1973), utilizes glucose as a major respiratory fuel and converts 60-70% of it into lactate and 25-30% into CO_2 (Suter and Weidemann, 1975). While most of the glycolytic activity (90%) has been attributed to the lymphocyte sub-population, the macrophages evidently account for a significant portion of the respiratory activity of the spleen (Suter and Weidemann, 1975). Substances such as pyruvate, acetoacetate and 3-hydroxybutyrate, although metabolized rapidly, are only 50% as effective as glucose in providing energy for the anabolic functions of lymphocytes (Nordeen and Young, 1977). Mitochondria isolated from spleen are capable of oxidizing long-chain fatty acids efficiently (see Chapter II), although added individual fatty acids such as sterate, oleate and palmitate contribute only 2% of the immediate respiratory fuel of the sliced tissue (D. Suter and M.J. Weidemann, unpublished observations). It was suggested, on the basis of the above observations, that exogenous labelled fatty acids are incorporated before oxidation into a general pool of triglyceride and subsequently liberated for oxidation at a greatly diluted specific radioactivity (Suter, 1973).

During long-term incubations *in vitro*, lymphoid tissues depend on the oxidation of endogenous amino acids derived from protein breakdown - as evidenced by an increase (45%) in the pool size of amino acids and the

liberation of free NH_4^+ (Suter and Weidemann, 1976; see Chapter II also). Adenosine has been suggested by Nordeen and Young (1977) as an alternate fuel to glucose. These workers observed that adenosine is able to restore energy-dependent functions of lymphocytes as well as enhancing the cellular glycogen stores. The rate of oxidation of [U-^{14}C]-adenosine to $^{14}CO_2$ is similar to that of glucose. Based on these results it has been suggested that adenosine substitutes for glucose by contributing its ribose moiety as an energy-providing substrate by the following pathway:

$$\text{adenosine} \xrightarrow{a} \underset{+\ NH_4^+}{\text{inosine}} \xrightarrow{b} \underset{+\ \text{hypoxanthine}}{\text{ribose-1-P}} \xrightarrow{c} \text{ribose-5-P}$$

where reaction "a" is catalyzed by adenosine deaminase; "b" by purine nucleoside phosphorylase; and "c" by phosphoribomutase (see Nordeen and Young, 1977). Identification of the enzymes involved in catalyzing the above reaction sequence in thymus (Manson and Lampen, 1951) gives additional support to this view. Further analysis showed that amino acids (glycogenic) and adenosine are both eventually converted into acetyl CoA before being oxidized to CO_2 by lymphoid tissues (see Suter and Weidemann, 1976; Nordeen and Young, 1977).

Rat thymus lymphocytes (10^{10} cells) metabolizing glucose (5mM), consumed about 412µ moles of O_2 during a 3 hr incubation period and formed 50µ moles of lactate plus pyruvate (see Table 2.1). The relative amounts of ATP produced by the cytosolic and mitochondrial processes can be calculated in the following way:

(a) cytosol

glucose ⟶ pyruvate + lactate

1 unit of glucose oxidation produces...2 units of ATP

∴ 50 units of lactate plus pyruvate (i.e., 25 units of glucose) produces } ..2 × 25 units of ATP

(b) mitochondrial:

pyruvate $\longrightarrow$ CO_2 + H_2O

1 unit of oxygen consumed in the above reaction produces..... 3 units of ATP
(since the ADP/O ratio for NAD-linked substrates is 3)

412 units of O_2 {i.e., 824 [O]} corresponds to 3 × 824 units of ATP

From the energetic point of view it is evident that more than 96% of the ATP is supplied by mitochondrial oxidative phosphorylation while less than 4% is formed by cytosolic substrate-level phosphorylation.

Addition of acetoacetate inhibits significantly (>90%) the flow of glucose carbon to CO_2 (i.e., the mitochondrial oxidation of pyruvate derived from glucose). However, it does not change the rate of consumption of oxygen, as acetoacetate itself is a preferred fuel of respiration. Secondly, the utilization of glucose by thymocyte glycolysis is not diminished by the presence of acetoacetate (see Table 2.3). In contrast, the enhanced metabolism of acetoacetate, free fatty acids or ketone bodies by many other tissues usually inhibits glycolysis through the production of increased steady-state levels of cytoplasmic citrate. Citrate is a negative allosteric effector of the low activity, non-equilibrium and (usually) rate-limiting enzyme (phosphofructo kinase) of the glycolytic pathway (Newsholme and Start, 1974). The absence of such a response to acetoacetate led Yasmeen *et al.* (1977) to propose that phosphofructokinase is not the flux-generating reaction for glycolysis in thymocytes (see Culvenor and Weidemann, 1976).

6.2 METABOLIC CHANGES DURING THE TRANSFORMATION OF LYMPHOCYTES

Energy-linked functions of lymphocytes can be supported either by glucose or a range of alternative oxidizable substrates (e.g., acetoacetate, pyruvate, etc.). However, their transformation to the blast stage depends exclusively on the metabolism of glucose (Nordeen and Young, 1977). The glycolytic pathway evidently serves important functions other than supplying acetyl CoA for energy production.

Transformation of lymphocytes by concanavalin A or A23187 involves a series of morphological and metabolic changes which culminate in cell division (Yasmeen *et al.*, 1977; see also Chapter II). Prominent among the early changes is an alteration in the permeability of the plasma membrane to metabolites such as sugars, nucleosides (Peters and Hausen, 1971) and amino acids (Mendelsohn *et al.*, 1971) and to ions such as P_i, K^+ and Ca^{2+} (Freedman *et al.*, 1975; see also Chapter I). Increased glucose uptake and a concomitant increase in lactate production has been shown to begin within 15 minutes of the stimulation of lymphocytes with phytohaemagglutinin (Peters and Hausen, 1971; Culvenor and Weidemann, 1976). However, lymphocytes are capable of undergoing transformation even when glucose is present in the medium at very low concentrations (25μM; Hume *et al.*, 1978). The K_m of the glucose carrier is much higher (0.32mM; A J. Laird, unpublished observations) than the concentration of glucose used in these experiments; secondly, concanavalin A addition does not alter the K_m for glucose (Yasmeen *et al.*, 1977); together, these findings suggest that the enhanced oxidation of glucose, as such, is not essential for the transformation of lymphocytes (e.g., initiation of DNA synthesis; Hume *et al.*, 1978). Similar results have been reported for proliferating fibroblasts (Naiditch and Cunningham, 1977). The results presented in Chapter II indicate that the increase in glucose uptake is accompanied by a commensurate increase in the incorporation of glucose carbon into

RNA. Biosynthesis of purine bases, which is stimulated in mitogen-treated lymphocytes (Hovi *et al.*, 1976), seems to depend on an enhanced rate of glucose uptake, as the rate of production of the precursor, phosphoribosyl-pyrophosphate (Green and Martin, 1973), depends on the availability of glucose-6 phosphate for entry into the oxidative segment of the pentose phosphate pathway (Culvenor and Weidemann, 1976; Hume *et al.*, 1978). It has therefore been suggested that the major role of increased aerobic glycolysis is to maintain higher steady-state levels of glycolytic intermediates that may, in turn, provide precursors for the synthesis of macromolecules during the transformation of lymphocytes (Hume *et al.*, 1978).

6.3 ROLE OF Ca^{2+} IN LYMPHOCYTE TRANSFORMATION

Studies on changes in glycolytic intermediates during mitogen-induced stimulation of lymphocytes led Culvenor and Weidemann (1976) to propose that activation of the glucose carrier, which is a low activity, non-equilibrium step anterior to phosphofructo kinase, is the earliest event in the activation of glycolysis. The conclusion that glucose transport across the plasma membrane is the rate-limiting step for the activity of the glycolytic pathway is based on the following observations:

(a) the mass action ratio of the glucose carrier is displaced from equilibrium by one order of magnitude (G. Keig and M.J. Weidemann, unpublished observations);

(b) the blood glucose concentration (6.7μ moles/ml of extracellular water) greatly exceeds the K_m for D-glucose (0.32mM; A.J. Laird, unpublished observations) in both stimulated and unstimulated cells;

(c) the catalytic capacity of the carrier is much lower than the maximum activities of the lowest activity enzymes (e.g., hexokinase and phosphofructo kinase) in the cytosol (Yasmeen *et al.*, 1977);

and, finally,

(d) the activation of the glucose carrier by concanavalin A produces a corresponding increase in lactate and CO_2 production, which is a characteristic criterion of a flux-generating step, i.e., changes in its activity produce, corresponding changes in pathway flux (Kaiser and Burns, 1973).

Similar conclusions have been reached by Whitesell *et al.* (1977).

Increased flux of exogenous Ca^{2+} into lymphocytes, which is one of the most prominent early events during mitogen stimulation (Freedman *et al.*, 1975), precedes the enhancement of various transport processes (e.g., α-aminobutyrate and glucose; Whitney and Sutherland, 1973; Yasmeen *et al.*, 1977; see also Chapter I for details). A working hypothesis has been proposed along the following lines by Weidemann and his co-workers in an attempt to explain how accelerated glucose uptake may correlate with Ca^{2+} entry. Lymphocytes produce reactive oxygen species (e.g., $^1O_2$, H_2O_2, $OH^{\cdot}$) within 30s after treatment with concanavalin A or A23187 that can be detected as chemiluminescence under appropriate conditions (Weidemann *et al.*, 1978). H_2O_2 has been suggested as the most probable species involved in generating the photons, as the chemiluminescence response to concanavalin A is sensitive to catalase and is dependent on the presence of easily-oxidizable substances such as luminol* in the medium. Since Ca^{2+} entry associated with lectin-binding (Freedman *et al.*, 1975) has a similar time-course to that of the emission of chemiluminescence, Ca^{2+} has been implicated in the production of H_2O_2. Ca^{2+} may act by enhancing the activity of a plasma-membrane-bound NAD(P)H oxidase by one of the following mechanisms: (a) Ca^{2+} ions, by virtue of their stimulatory action on glycogenolysis (Newsholme and Start, 1974), may increase the supply of

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Luminol* = 5-amino-2, 3-dihydro-1,4 phthalazinedione.

NAD(P)H for the oxidase; or (b) Ca^{2+} ions may be involved in the stimulation of the membrane bound NAD(P)H oxidase through activation of Ca^{2+}-sensitive phospholipase A_2 (Pickett *et al.*, 1977; Weidemann *et al.*, 1978) and the subsequent alteration in the composition of specific phospholipids present in the plasma membrane. The production of H_2O_2, which is itself a mitogenic compound for T-lymphocytes, may then be involved in the enhancement of glucose transport (Weidemann *et al.*, 1978). Since addition of sulphydryl-blocking reagents, such as N-ethyl maleimide, block the concanavalin A-stimulated phase of glucose uptake, it has been suggested that the formation of a disulphide bridge between two inactive monomeric forms of the glucose carrier transform it into an active state (Czech, 1975; Hume and Weidemann, 1978). Endogenously produced H_2O_2 (or any other suitable oxidizing agent) may be able, in the presence of an appropriate enzyme, to dimerize the inactive carrier molecules to their active form. Thus, a small increase of Ca^{2+} ion concentration in the cytoplasm of lymphocytes (as a result of lectin-binding) that mobilizes glycogen and produces H_2O_2 (through NADPH supply to the membrane-bound NADPH-oxidase) has the potential to trigger the stimulation of glucose transport.

A sequence of events similar to those described above that lead to the activation of glucose transport has already been established in adipose tissue treated with insulin (Mukherjee and Lynn, 1977). This may be a general mechanism of cell activation shared by anabolic hormones, antigens and the virus-induced transformation of mammalian cells where DNA precursors are required for transformation and cell division (Hume *et al.*, 1978).

In addition to its possible role in the regulation of the glucose carrier, an increase in cytoplasmic free Ca^{2+} in the nanomolar range (see Chapter II) is apparently sufficient to account for the stimulation of pyruvate oxidation observed in whole thymus or spleen cells treated

with concanavalin A or A23187. The underlying mechanism of activation of pyruvate dehydrogenase by Ca^{2+} ions has been discussed earlier (Chapters I and II). Thus, a specific increase in the oxidation of pyruvate by Ca^{2+}-ions enhances the percentage contribution of glucose to the respiratory fuel by 30-40%. This provides energy that alternate or endogenous substrates are insufficient to supply during the 72 hour period of lymphocyte transformation. However, when rat thymus lymphocytes are treated with A23187 there is a gradual decline in the rate of oxygen consumption during the 3hr incubation (M.J. Weidemann, unpublished observations). The above observation is consistent with the inhibitory effects of high concentrations of Ca^{2+} (>50nM) on the oxidation of NAD-linked substrates (see Chapter II). The uncontrolled flux of Ca^{2+} into the cytoplasm of lymphocytes in the presence of A23187 could elevate the free Ca^{2+} level above 50nM (L.M. Russell, unpublished observations). The results suggest, from a physiological view-point, an upper limit of 50nM for the free Ca^{2+} concentration in the cytosol, above which there is irreversible damage to mitochondrial function (see also Chapter II).

6.4 REGULATION OF CYTOSOLIC Ca^{2+}

In view of the importance of Ca^{2+} in a range of lymphocyte functions, an efficient mechanism for the regulation of its cytosolic free concentration might be anticipated. The amount of free Ca^{2+} in the cytoplasm of any tissue usually constitutes only a small fraction of the total cellular Ca^{2+} (Bygrave, 1977), the majority of which is retained mainly in mitochondria, the endoplasmic/sarcoplasmic reticulum and, in a bound form, by various membranes. The cytosolic level is determined by the relative rates at which Ca^{2+} is accumulated and released from intracellular pools as well as by its net transport across the plasma membrane (see Chapter I for details). The regulatory mechanisms for the maintenance of Ca^{2+} homeostasis in the cytoplasm of lymphocytes is not known. Available

evidence suggests that lymphocytes have a limited number of Ca^{2+}-sequestering organelles such as mitochondria and endoplasmic reticulum vesicles. Moreover, since no information is available on the ability of the lymphocyte plasma membrane to regulate cytoplasmic Ca^{2+} levels, it has been suggested that mitochondria may play a major role in determining its cytosolic level (see Chapter V).

Preliminary studies (Chapter V) have indicated that spleen mitochondria are less efficient than those of liver in their ability to transport Ca^{2+}. For instance, at 0°C, spleen mitochondria accumulate Ca^{2+} at a very low rate. However, a dramatic increase (2.5 fold) in the rate of Ca^{2+} transport by spleen mitochondria has been observed in the presence of Mg^{2+} and ATP (see Chapter V). Since this observation has only been made with spleen and hog heart mitochondria (Le Blanc and Clauser, 1974), the possible mechanisms that could lead to the enhanced uptake of Ca^{2+} have been investigated. The results presented in Chapter V indicate that the outer membranes of spleen mitochondria may have selective permeability properties and suggest that there could be a distinct metabolite pool present in the inter-membrane space. The outer membrane ATPase may control the movement of Ca^{2+} between the inter-membrane space and the extramitochondrial compartment (stage 1), whereas the movement of Ca^{2+} across the inner membranes (stage 2) is dependent on the activity of the proton-motive-force-linked Ca^{2+} transporter. According to this hypothesis, Ca^{2+}-ions could be stored temporarily in the inter-membrane space. This would enable mitochondria to sequester the elevated concentration of cytosolic Ca^{2+} that occurs immediately after lectin binding. It is worth noting in this context that the gated flux of Ca^{2+} into the cytoplasm that occurs for only 40s after lectin-binding is small (Freedman *et al.*, 1975). Moreover, the relative distribution of Ca^{2+} transported into mitochondria into the inter-membrane and matrix space is not clearly known, although preliminary results indicate that a major proportion of Ca^{2+} may

be retained between the two membranes (see Chapter V). It is reasonable to assume that the flux of Ca^{2+} into the matrix space, under these conditions does not exceed 50nM as this would otherwise prevent the oxidation of NAD-linked substrates (see Chapter II); since the oxidation of glucose (via pyruvate) is enhanced in concanavalin A-treated lymphocytes, it is unlikely that the matrix Ca^{2+} concentration increases excessively.

Outer membranes of mitochondria have more properties in common with the membranes of the sarcoplasmic reticulum than with the inner mitochondrial membranes (see Chapter I). Thus, the outer membranes of mitochondria may have functions in non-muscle cells, such as Ca^{2+}-transport, similar to those of the sarcoplasmic reticulum. This property may be important in sperm mitochondria, for example, as the motility of sperm is dependent on short-term fluxes of Ca^{2+}, that require an efficient means of cyclically regulating the cytosolic Ca^{2+} concentration. Inhibition of the motility of sperm by Cu^{2+} (D. Suter and I. White, unpublished observations) may be explained in terms of a Cu^{2+}-induced inhibition of mitochondrial Ca^{2+} transport by a mechanism similar to that described for spleen mitochondria (see Chapter V). Similar mechanisms may be anticipated in mitochondria from the macrophage sub-population of spleen that is involved in phagocytic movements during particle ingestion. This mechanism may also be pertinent to heart muscle contractile events where an influx of Ca^{2+} from the extracellular space needs to be sequestered efficiently in the sarcoplasm. As heart cells do not have a fully-developed sarcoplasmic reticulum, mitochondria and sarcolemma have been implicated in regulating the sarcoplasmic Ca^{2+} level (see Chapter I). The participation of the outer membranes of heart mitochondria may enhance their ability to regulate Ca^{2+} levels efficiently. Identification of an ATPase in the atractyloside-insensitive space of heart mitochondria is consistent with this view.

6.5 FUTURE WORK

This work raises a number of unresolved questions. For instance, identification of the oligomycin-insensitive ATPase as an outer membrane enzyme of spleen mitochondria raises the question of the nature of the lymphoid cell type that possesses it. Preliminary studies have shown that the ATPase could be a marker enzyme for mitochondria from the B-cell sub-population of spleen (U. Hurtenbach and M.J. Weidemann, unpublished observations). There is however, a low but finite activity of the oligomycin-insensitive ATPase in splenic T-cell sub-populations, which is consistent with the observation that thymus mitochondria contain measurable activity at 30°C (see Chapter III). Since these observations are incomplete, this work may be extended further by establishing the exact nature of the sub-populations of lymphoid cells that contain the ATPase.

More importantly, the results presented in Chapter III indicate that mitochondria from several other tissues (e.g., heart and kidney-cortex) may have bivalent cation-dependent ATPases in their outer membranes. Some evidence is available to support a role for the outer membrane ATPase of kidney-cortex mitochondria in the regulation of Ca^{2+} fluxes (Gmaj *et al.*, 1974), but this has not been proven conclusively. Substantial differences exist between heart and liver mitochondria with respect to their membrane permeability properties (Brierley *et al.*, 1970; Gamble and Lehninger, 1973), enzymic capacity (Jacobus and Lehninger, 1973) and energy-linked accumulation of Ca^{2+} (Jacobus *et al.*, 1975). However, a specific impermeability of the outer membranes to low molecular weight substances has not been established. The results presented in Chapter V provide some evidence to support the hypothesis that the outer membranes of spleen mitochondria may be relatively impermeable to Ca^{2+} ions; but they do not explain the direct accessibility of exogenously added inhibitors

such as atractyloside to the carriers of the inner membranes. Further work is necessary to locate specific carriers for various metabolites in order to establish conclusively that the outer membranes of lymphoid tissue mitochondria have distinctive permeability properties. It was not possible to attempt to define such properties during this project because of the low yield of outer membranes obtained from rat spleen mitochondria. Similar studies could be done with pig spleen mitochondria where the yield of the enzyme is much larger and where purification could be undertaken.

A naturally occurring inhibitor of the ATP-synthase of bovine heart mitochondria has been purified (Pullman and Monroy, 1963; Horstman and Racker, 1970). It inhibits the ATP-hydrolyzing activity of sub-mitochondrial particles and the activity of the purified soluble ATPase of mitochondria strongly (van de Stadt *et al.*, 1973) but it does not inhibit oxidative phosphorylation (Asami *et al.*, 1970) or the ATP-dependent reduction of NAD^+ by succinate (Lang and Racker, 1974). No reports are available on the nature of inhibitors for other membrane-bound ATPases (e.g., ATPases of sarcoplasmic reticulum and plasma membrane). Initial attempts to identify an inhibitor protein molecule or a metabolite that could regulate the outer membrane ATPase were unsuccessful. More systematic attempts could be be made to isolate and purify regulator molecules from spleen by following the protocol used for the isolation of the ATP-synthase inhibitor protein from other tissues. Isolation of such a regulator molecule would throw more light on the possible functional role of the enzyme in the regulation of Ca^{2+} homeostasis.

BIBLIOGRAPHY

BIBLIOGRAPHY

Adolfsen, R. & Moudrianakis, E.N. (1973) *Biochemistry* 12, 2926 - 2933.

Agarwal, B.B.L. & Goldstein, I.J. (1967) *Biochim. biophys. Acta* 147, 267 - 271.

Alberty, R.A., Massey, V., Frieden, C. & Fuhlbrigge, A.R. (1954) *J. Am. Chem. Soc.* 76, 2485 - 2493.

Albrecht, S.P.J. & Slater, E.C. (1971) *Biochim. biophys. Acta* 245, 503 - 507.

Allmann, D.W., Bachmann, E. & Green, D.E. (1966) *Arch. Biochem. Biophys.* 115, 165 - 171.

Arcos, J.C., Stacey, R.E., Mathison, J.B. & Argus, M.F. (1967) *Exp. Cell Res.* 48, 448 - 460.

Arsenis, C., Maniatis, T. & Touster, O. (1968) *J. Biol. Chem.* 243, 4396 - 4399.

Asami, K., Juntti, K. & Ernster, L. (1970) *Biochim. biophys. Acta* 205, 307 - 311.

Ashwell, G. & Hickman, J. (1952) *Proc. Soc. Expl. Biol. Med.* 80, 407 - 410.

Azzi, A., Gherardini, P. & Santato, M. (1971) *J. Biol. Chem.* 246, 2035 - 2042.

Azzone, G.F. & Carafoli, E. (1960) *Exp. Cell Res.* 21, 447 - 467.

Baginsky, M.L. & Hatefi, Y. (1968) *Biochem. Biophys. Res. Commun.* 32, 945 - 950.

Baker, P.F., Blaustein, M.P., Hodgkin, A.L. & Steinhardt, R.A. (1969) *J. Physiol.* 200, 431 - 458.

Barlogie, B., Hasselbach, W. & Makinose, M. (1971) *FEBS Lett*, 12, 267 - 268.

Bartley, W. & Dean, B. (1968) *Anal. Biochem.* 25, 99 - 108.

Baugh, R.F. & King, T.E. (1972) *Biochem. Biophys. Res. Commun.* 49, 1165 - 1173.

Beaver, B. (1975) *B.Sc. (Hons) Thesis*, Australian National University.

Beechey, R.B., Roberton, A.M., Holloway, C.T. & Knight, I.G. (1967) *Biochemistry*. 6, 3867 - 3879.

Bergmeyer, H.U. & Bernt, E. (1965) in *"Methods of Enzymatic Analysis"* (Bergmeyer, H.U., Ed.) pp 384 - 388, Academic Press, New York.

Bergmeyer, H.U., Gawehn, K. & Grassl, M. (1974) in *"Methods of Enzymatic Analysis"* (Bergmeyer, H.U., ed.), Vol. 1, pp. 486 - 487, Academic Press, New York.

Beyer, R.E. (1960) *Biochim. biophys. Acta* 41, 552 - 553.

Bielawski, J. & Lehninger, A.L. (1966) *J. Biol. Chem.* 241, 4316 - 4322.

Blaustein, M.P. & Hodgkin, A.L. (1969) *J. Physiol. (London).* 200, 497 - 527.

Blondin, G. (1974) *Ann. N.Y. Acad. Sci.*, 227, 392 - 397.

Bonner, W.D. (1967) in *"Methods in Enzymol"*, 10, 126 - 133.

Borle, A. (1967) *Clin. Orthop.* 52, 267.

Borle, A. (1974) *J. Membr. Biol.* 16, 221 - 236.

Brierley, G.P., Murer, E. & Bachman, E. (1964) *J. Biol. Chem.* 239, 2706 - 2712.

Brierley, G.P. & Knight, V.A. (1967) *Biochemistry* 6, 3892 - 3902.

Brierley, G.P., Knight, V.A. & Settlemire, C.T. (1968) *J. Biol. Chem.* 243, 5035 - 5043.

Brierley, G.P., Jurkowitz, M., Scott, K.M. & Merola, A.J. (1970). *J. Biol. Chem.* 245, 5404 - 5411.

Bücher, T., Czok, R., Lamprecht, W. & Latzko, E. (1965) in *"Methods of Enzymatic Analysis"* (Bergmeyer, H.U., ed.), pp 253 - 259, Academic Press, New York.

Bulos, B. & Racker, E. (1968) *J. Biol. Chem.* 243, 3891 - 3900.

Bygrave, F.L. (1966) *Biochem. J.* 101, 488 - 491.

Bygrave, F.L. (1967) *Nature* (London) 214, 667 - 671.

Bygrave, F.L. (1975) *Fed. Eur. Biochem. Soc. Meet*, 10th, Abstract 1131.

Bygrave, F.L. (1976) In *"An Introduction to Bio-Organic Chemistry"*. (D.R. Williams, ed), pp 171 - 186. Thomas, Springfield, Illinois.

Bygrave, F.L., Daday, A.A., & Doy, F.A. (1975). *Biochem. J.* 146, 601 - 608.

Bygrave, F.L. (1977) in *"Current Topics in Bioenergetics"*, Vol. 6, D.R. Sanadi (ed)., pp 260 - 318. Academic Press, New York.

Capaldi, R.A. & Green, D.E. (1972) *FEBS Lett.*, 25, 205 - 209.

Capaldi, R.A. & Hayashi, H. (1972) *FEBS Lett*, 26, 261 - 263.

Capaldi, R.A. (1977) in *"Mammalian Cell Membranes"*. Vol. 2, pp 140 - 164., Jameison, G.A. & Robinson, D.M. (eds)., Butterworths, London.

Caplan, A.I. & Greenawalt, J.W. (1966) *J. Cell Biol.* 31, 455.

Carafoli, E. (1973). *Biochimie.* 55, 755 - 762.

Carafoli, E. (1974). *Biochem. Soc. Symp.* 39, 89 - 109.

Carafoli, E., & Sottocasa, G.L. (1974) In *"Dynamics of Energy - Transducing Membranes"* (L. Ernster, R.W., Estabrook & E.C. Slater, eds.), 455 - 469, Elsevier, Amsterdam.

Carafoli, E., Tiozzo, R., Lugli, C., Crovetti, F. & Kratzing, C.C. (1974) *J. Mol. Cell. Cardiol.* 6, 361 - 371.

Carafoli, E., Dabrowska, R., Crovetti, F., Tiozzo, R. & Drabikowski, W. (1975) *Biochem. Biophys. Res. Commun.* 62, 908 - 912.

Cardona, E., Lessler, M. & Brierley, G.P. (1971) *Proc. Soc. Exp. Biol. Med.* 136, 300 - 304.

Cassady, W.E. & Wagner, R.P. (1971). *J. Cell. Biol.* 49, 536 - 541.

Catterall, W.A. & Pedersen, P.L. (1971). *J. Biol. Chem.* 246, 4987 - 4994.

Catterall, W.A. & Pedersen, P.L. (1972) *J. Biol. Chem.* 247, 7969 - 7976.

Cederbaum, A.I. & Wainio, W.W. (1970) *Fed. Proc.* 29, p 864 (Abs. 3508).

Chao, D.L.S. & Davis, E.J. (1972) *Biochemistry.* 11, 1943 - 1952.

Chance, B. & Baltschefsky, M. (1958) *Biochem. J.* 68, 283 - 295.

Chance, B. & Packer, L. (1958) *Biochem. J.* 68, 295.

Chance, B. & Williams, G.R. (1956) *Adv. Enzymol.* 17, 65 - 134.

Chance, B. (1965) *J. Biol. Chem.* 240, 2729 - 2748.

Chance, B., Wilson, D.F., Dutton, P.L. & Erecinska, M. (1970) *Proc. Natl. Acad. Sci. U.S.A.* 66, 1175.

Chan, P.W.P. (1972). *B.Sc. (Hons) Thesis,* Australian National University.

Chappell, J.B. (1968) *Brit. Med. Bull.* 24, 150 - 157.

Claude, A. (1946). *J. Exptl. Med.* 84, 51 - 61.

Cleland, K.W. & Slater, E.C. (1953) *Biochem. J.* 53, 547 - 556.

Colbeau, A., Nachbaur, J. & Vignais, P.M. (1971) *Biochim. biophys. Acta,* 419, 271 - 284.

Cole, J.L., Fishler, M.C., Ellis, M.E. & Bond, V.P. (1952) *Proc. Soc. Exp. Biol. Med.* 80, p. 112.

Colli, W., Hinkle, P., & Pullman, M.E. (1968). *Fed. Proc.* 27, p 648.

Comte, J. Maisterrena, B. & Gautheron, D.C. (1976). *Biochim. biophys. Acta.* 419, 271 - 284.

Cooper, C. & Lehninger, A.L. (1957) *J. Biol. Chem.* 224, 547 - 560.

Culvenor, J.G. & Weidemann, M.J. (1976). *Biochim. biophys. Acta.* 437, 354 - 363.

Czech, M.P. (1975) *J. Cell. Physiol.* 89, 661.

Danielli, J.F. & Davson, H. (1935) *J. Cell. Comp. Physiol.* 5, 495.

Davies, K.A. & Hatefi, Y. (1971) *Biochemistry,* 10, 2509 - 2516.

Davies, K.A., Hatefi, Y., Paff, K.L. & Butler, W.L. (1972) *Biochem. Biophys. Res. Commun.*, 46, 1984 - 1990.

Day, D.A., & Wiskich, J.T. (1975) *Arch. Biochem. Biophys.* 171, 117 - 123.

De Luca, H.F. & Engstrom, G.W. (1961) *Proc. Natl. Acad. Sci. U.S.A.* 47, 1744 - 1750.

Denton, R.M., Randle, P.J. & Martin, B.R. (1972) *Biochem. J.* 128, 161 - 163.

Denton, R.M., Randle, P.J., Bridges, B.J., Cooper, R.H., Kerbey, A.L., Pask, H.T., Severson, D.L., Stansbie, D. & Whitehouse, S. (1975) *Mol. Cell Biochem.* 9, 27 - 53.

Dietze, G. & Hepp, K.D. (1971) *Biochem. Biophys. Res. Commun.* 44, 1041.

Dietze, G. & Hepp, K.D. (1972) *Biochem. Biophys. Res. Commun.* 46, 269 - 277.

Dickerson, R.E., Takano, T., Eisenberg, D., Kallai, A., Sampson, L., Cooper, A. & Margoliash, E. (1971) *J. Biol. Chem.* 246, 1511 - 1535.

Dixon, M. & Webb, E.C. (1964) in *Enzymes,* pp. 315 - 359, Longmans, Green & Co., London.

Douce, R., Guillot-Salomon, R., Lance, C. & Signol, M. (1968). *Bull. Soc. Franc. Physiol. Vé gét.* 14, 351 - 373.

Douglas, W.W. & Rubin, R.P. (1961) *J. Physiol.* 159, 40 - 57.

Dulley, J.R. (1975) *Anal. Biochem.* 67, 91 - 96.

Ebashi, S. & Lipmann, F. (1962) *J. Cell. Biol.* 14, 389 - 400.

Ebashi, S., Endo, M. & Ohtsuki, I. (1969) *Quart. Rev. Biophys.* 2, 351 - 384.

Ebel, R.E. & Landy, H.A. (1975) *J. Biol. Chem.* 250, 191 - 196.

Eichel, H.J. (1957) *J. Biophys. Biochem. Cytol*, 3, 397 - 412.

Entman, M.L., Levey, G.S. & Epstein, S.E. (1969) *Circ Res* 25, 429 - 438.

Ernster, L. & Kuylenstierna, B. (1968) in *"Mitochondria - Structure and function"* Ernster, L. & Kuylenstierna (eds), Proc. 5th FEBS Meeting, Prague, Publ., Czechosl. Acad. Sci., Prague, and Academic Press, London.

Ernster, L. & Kuylenstierna, B. (1970) in *"Membranes of Mitochondria and Chloroplasts"* (Racker, E., ed.), A.C.S. Monograph 165, pp. 172 - 206, Van Nostrand-Reinhold Co., New York.

Farstad, M., Bremer, J. & Norum, K.R. (1967). *Biochim. biophys. Acta.* 132, 492 - 502.

Fergusen, S.J., Harris, D.A. & Radda, G.K. (1977) *Biochem. J.* 162, 351 - 357.

Fernandez-Moran, H. (1962) *Circulation*, 26, 1039 - 1065.

Fisher, A.B., Scarpa, A., La Noue, K.F., Basselt, D. & Williamson, J.R. (1973) *Biochemistry* 12, 1438 - 1445.

Fiske, C.H. & Subbarow, Y. (1925) *J. Biol. Chem.* 66, 375 - 400.

Foldes, M. & Barritt, G.J. (1977) *J. Biol. Chem.* 252, 5372 - 5380.

Forster, G., Bernt, E. & Bergmeyer, H.U. (1974) in *"Methods of Enzymatic Analysis"* (Bergmeyer, H.U., ed.), Vol. 2, pp. 785 - 793, Academic Press, New York.

Freedman, M.H., Raff, M.C. & Gomperts, B. (1975) *Nature*, 255, 378 - 382.

Frieden, C. (1964) *J. Biol. Chem.* 239, 3522 - 3531.

Friedman, Z. & Makinose, M. (1970) *FEBS Lett.* 11, 69 - 72.

Gamble, J.G., & Lehninger, A.L. (1973). *J. Biol. Chem.* 248, 610 - 618.

Garrahan, P.J. & Glynn, I.M. (1967) *J. Physiol. (Lond.)*. 192, 217 - 235.

Gevers, W. & Krebs, H.A. (1966) *Biochem. J.* 98, 720 - 735.

Glauert, A.M. (1975) in *"Practical Methods in Electron Microscopy"*. Vol 3., Part I, North Holland Publishing Co., Amsterdam.

Glynn, I.M. (1956) *J. Physiol. (Lond)* 134, 278 - 310.

Glynn, I.M. & Chappell, J.B. (1964) *Biochem. J.* 90, 147 - 149.

Glynn, I.M. (1968) *Brit. Med. Bull.* 24, 165 - 169.

Gmaj, P., Nowicka, C. & Angielski, S. (1974) *FEBS Lett.* 47, 76 - 80.

Gomperts, B.D. (1976) in *Receptors and Recognition"*, Series A, Vol. 2, pp 45 - 93 Cuatrecasas, P. & Greaves, M.F. (eds) Publ:

Chapman & Hall, London.

Graham, J.M. & Green, C. (1970) *Eur. J. Biochem.* 12, 58 - 66.

Green, D.E., Bachmann, E., Allmann, D.W. & Perdue, J.F. (1966) *Arch. Biochem. Biophys.* 115, 172 - 180.

Greene, W.C., Parker, C.M. & Parker, C.W. (1976) *Cell. Immunol* 25, 27 - 89.

Greene, C.D. & Martin, D.W. Jr. (1973) *Cell* 2, 241 - 245.

Grisham, C.M. & Barnett, R.E. (1973) *Biochemistry.* 12, 2635 - 2637.

Grisolia, S. & Mendelson, J. (1974) *Biochem. Biophys. Res. Commun.* 58, 968 - 973.

Gunther, T. (1967) *Z. Naturforsch,* 22b, 149.

Hachimori, A., Muramatsu, N. & Nosoh, Y. (1970) *Biochim. biophys. Acta* 206, 426 - 437.

Hackenbrock, C.R. (1966). *J. Cell. Biol.* 30, 269 - 297.

Halestrap, A.P. & Denton, R.M. (1974) *Biochem. J.* 138, 313 - 316.

Halestrap, A.P. (1975). *Biochem. J.* 148, 85 - 96.

Hanson, R.L. & Kennedy, E.P. (1973) *J. Bacteriol.* 114, 772 - 781.

Harris, E.J. (1972). *J. Mem. Biol.* 9, 141 - 154.

Harris, D.A., Rosing, J., Van de Stadt, R.J. & Slater, E.C. (1973) *Biochim. biophys. Acta* 314, 149 - 153.

Haslam, R.J. & Krebs, H.A. (1963) *Biochem. J.* 88, 566 - 578.

Hasselbach, W. & Makinose, M. (1961). *Biochem. Z.* 333, 518 - 528.

Hasselbach, W. & Makinose, M. (1963) *Biochem. Z.* 339, 94 - 111.

Hasselbach, W. (1964). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* 23, 909 - 912.

Hasselbach, W. (1974). in *"The Enzymes"*, Vol. X, 3rd Edition, pp 413 - 467.

Hatefi, Y. (1973) *Biochem. Biophys. Res. Commun.*, 50, 978 - 984.

Hayashi, H. & Capaldi, R.A. (1972) *Biochim. biophys. Acta,* 282, 166 - 173.

Hedeskov, C.J. (1968) *Biochem. J.* 110, 373 - 380.

Heldt, H.W. & Schwalbach, K. (1967). *Eur. J. Biochem.* 1, 199 - 206.

Hemker, H.C. (1963). *Biochim. biophys. Acta* 73, 311 - 323.

Hesketh, T.R., Smith, G.A., Houslay, M.D., Warren, G.B. & Metcalfe, J.C. (1977). *Nature*, 267, 490 - 494.

Hexter, C.S. & Goldman, K. (1973) *Biochem. biophys. Acta*, 307, 421 - 427.

Hickman, P.E. (1974) *Ph.D. Thesis*, Australian National University.

Higgins, J.A. & Barrnett, R.J. (1970) *J. Cell. Sci.* 6, 29 - 51.

Hilden, S., Rhee, H.M. & Hokin, L.E. (1974) *J. Biol. Chem.* 249, 7432 - 7440.

Hohorst, H.J. (1965) in *"Methods of Enzymatic Analysis"* pp 260 - 270.

Hokin, L.E., Dahl, J.L., Deupree, J.D., Dixon, J.F., Hackney, J.F. & Perdue, J.F. (1973), *J. Biol. Chem*, 248, 2593 - 2605.

Horstman, L.L. & Racker, E. (1970) *J. Biol. Chem.* 245, 1336 - 1344.

Hoskins, D.D. & Mackenzie, C.G. (1961) *J. Biol. Chem*, 236, 177 - 183.

Hovi, T., Smyth, J.F., Allison, A.C. & Williams, S.C. (1976) *Clin. Exp. Immunol.* 23, 395 - 403.

Hume, D.A. & Weidemann, M.J. (1978) *J. Cell. Physiol* (in Press).

Hume, D.A., Radik, J.L., Ferber, E. & Weidemann, M.J. (1978) *Biochem. J.* (in Press).

Hume, D.A., Vijayakumar, E.K., Schweinberger, F., Russell, L.M. & Weidemann, M.J. (1978). *Biochem. J.* (in Press).

Hwang, K.M., Scott, K.M. & Brierley, G.P. (1972) *Arch. Biochem. Biophys.* 150, 746 - 756.

Jacobson, L.O. (1952) *Cancer Research*, 12, 315 - 323.

Jacobs, H., Heldt, H.W. & Klingenberg, M. (1964) *Biochem. Biophys. Res. Commun.* 16, 516 - 521.

Jacobus, W.E. & Brierley, G.P. (1969) *J. Biol. Chem.* 244, 4995 - 5004.

Jacobus, W.E. & Lehninger, A.L. (1973) *J. Biol. Chem.* 248, 4803 - 4810.

Jacobus, W.E., Tiazzo, R., Lugli, G., Lehninger, A.L. & Carafoli, E. (1975) *J. Biol. Chem.* 250, 7863 - 7870.

Jagow, G.V., Westermann, B. & Wieland, O. (1968). *Eur. J. Biochem.* 3, 512 - 518.

Janke, J., Oberdisse, A. & Petzoldt, C. (1970). *Pflueger. Arch.* 314, 124 - 140.

Jaworek, D., Grauber, W. & Bergmeyer, H.U. (1974) in *"Methods in Enzymatic Analysis"* Vol. 4., pp 2127 - 2131.

Jensen, P. & Rasmussen, H. (1977) *Biochim. biophys. Acta*, 468, 146 - 156.

Johnson, D. & Lardy, H. (1967) in *"Methods Enzymol."* Vol. 10, 94 - 96.

Jones, M.S. & Jones, O.T.G. (1968) *Biochem. Biophys. Res. Commun*, 31, 977 - 982.

Jorgensen, P.L. & Skou, J.C. (1971) *Biochim. biophys. Acta.* 233, 366 - 380.

Jost, P., Griffith, O.H., Capaldi, R.A. & Vanderkooi, G. (1973) *Biochim. biophys. Acta*, 311, 141 - 152.

Kagawa, Y. & Racker, E. (1966) *J. Biol. Chem*, 241, 2461 - 2466.

Kagawa, Y. & Racker, E. (1971) *J. Biol. Chem.* 246, 5477 - 5487.

Kagawa, Y., Kandrach, A. & Racker, E. (1973). *J. Biol. Chem.* 248, 676 - 684.

Kagawa, Y. (1976). *J. Cell. Physiol.* 89, 569 - 574.

Kagawa, Y., Sone, N., Yoshida, M., Hirata, H. & Okamoto, H. (1976). *J. Biochem (Tokyo)*, 80, 141 - 151.

Kacser, H. & Burns, J.A. (1973) in *"Rate Control of Biological Processes"* (Davies, D.D., ed), Symposia of the Society for Experimental Biology XXVII, pp. 65 - 104, Cambridge University Press.

Katz, A.M., Repke, D.I., Dunnett, J. & Hasselbach, W. (1977a). *J. Biol. Chem.* 252, 1950 - 1956.

Katz, A.M., Repke, D.I. & Hasselbach, W. (1977b) *J. Biol. Chem.* 252, 1938 - 1949.

Kennedy, E.P. & Lehninger, A.L. (1949) *J. Biol. Chem*, 179, 957 - 972.

Kinne-Saffran, E. & Kinne, R. (1974) *J. Membr. Biol.* 17, 263 - 274.

Kitiakowsky, G.B. & Rosenberg, A.J. (1952) *J. Am. Chem. Soc.* 74, 5020 - 5025.

Klingenberg, M. (1972) in *"Mitochondrial Biomembranes"*, Proceedings of the 8th FEBS Meeting in Amsterdam, pp. 147 - 162. Elsevier, Amsterdam.

Klingenberg, M. (1975) in *"The Enzymes of Biol. Memb."* Vol. 3, pp 383 - 438.

Komai, H. & Capaldi, R.A. (1973) *FEBS Lett*, 30, 273 - 276.

Koshland, D.E., Jr. (1970) in *"Enzymes"* 2nd Ed. 1, 341 - 346.

Krebs, H.A. & de Gasquet, P. (1964) *Biochem. J.* 90, 149 - 154.

Krebs, H.A., Hems, R., Weidemann, M.J. & Speake, R.N. (1966) *Biochem. J.* 101, 242 - 249.

Kropp, E.S. & Wilson, J.E. (1970) *Biochem. Biophys. Res. Commun,* 38, 74 - 79.

Kun, E. (1976) *Biochemistry,* 15, 2328 - 2336.

Kubayoma, M., Fong, F.C. & King, T.E. (1972) *J. Biol. Chem.* 247, 6375 - 6383.

Lamprecht, W. & Trautschold, T. (1974) in *"Methods of Enzymatic Analysis"* Vol. 4., pp 2101 - 2110.

Laird, A.J. (1974) *B.Sc. (Hons). Thesis,* Australian National University.

Lang, D.R. & Racker, E. (1974) *Biochem. biophys. Acta* 333, 180 - 186.

La Raia, P.J. & Morkin, E. (1974) *Circ. Res.* 35, 298 - 306.

Lardy, H.A., Johnson, D. & McMurray, W.C. (1958) *Arch. Biochem. Biophys.* 78, 587 - 597.

Layne, E. (1957) in *"Methods in Enzymology"* 3, 450 - 451.

Le Blanc, P. & Clauser, H. (1974) *Biochem. biophys. Acta,* 347, 87 - 101.

Lee, C.P. & Ernster, L. (1966) in *Regulation of Metabolic Processes in Motichondria* B.B.A. Library, Vol. 7, p 218 Tager, J.M., Papa, S., Quagliariello, E. & Slater, E.C. (eds) Elsevier Pub. Co., Amsterdam.

Lee, C.P. & Ernster, L. (1967) in *Methods in Enzymol,* 10, 543 - 548.

Lehninger, A.L. (1962) *Physiol. Rev.* 42, 467 - 517.

Lehninger, A.L. (1971) *Biochem. Biophys. Res. Commun.* 42, 312 - 318.

Lehninger, A.L. (1973) *Neurosci. Res. Prog. Bull.* 11, 185 - 192.

Lehninger, A.L., Carafoli, E. & Rossi, C.S. (1967). *Adv. Enzymol. Relat. Areas Mol. Biol.* 29, 259 - 320.

Levy, M. & Sauner, M.T. (1967) *Compt. Rend. Soc. Biol.* 161, 277 - 279.

Levy, M., Toury, R. & Andre, J. (1967) *Biochim. biophys. Acta* 135, 599 - 613.

Lieberman, I., Lansing, A.I. & Lynch, W.E. (1967). *J. Biol. Chem.* 242, 736 - 739.

Lima, M.S., Nachbaur, G. & Vignais, P.B. (1968). *C. r. hebd. Seanc. Acad. Sci.* Paris, D. 266, 739 - 742.

Linn, T.C., Pettit, F.H., Hucho, F. & Reed, L.J. (1969a) *Proc. Nat. Acad. Sci. (USA)* 62, 227 - 233.

Linn, T.C., Pettit, F.H., Reed, L.J. (1969b) *Proc. Nat. Acad. Sci. (USA)* 62, 234 - 241.

Loyter, A., Christiansen, R.O., Steensland, H., Saltzgaber, J. & Racker, E. (1969). *J. Biol. Chem.* 244, 4422 - 4427.

Lowry, O.H.W.H. (1957) in *"Methods in Enzymol.* 4, 373 - 374.

Luckasen, J.R., White, J.G. & Kersey, J.H. (1974) *Proc. Nat. Acad. Sci. (USA).* 71, 5088 - 5090.

MacLennan, D.H. & Tzagoloff, A. (1968) *Biochemistry,* 7, 1603 - 1610.

MacLennan, D.H. & Wong, P.T.S. (1971) *Proc. Natl. Acad. Sci. U.S.A.* 68, 1231 - 1235.

MacLennan, D.H., Yip, C.C., Iles, G.H. & Seeman, P. (1972). *Cold. Spring Harbour Symp. Quant. Biol.* 37, 469 - 478.

MacLennan, D.H. (1970). *J. Biol. Chem.* 245, 4508 - 4518.

MacLennan, D.H. (1974). *J. Biol. Chem.* 249, 980 - 984.

Maino, V.C., Green, N.M. & Crumpton, M.J. (1974) *Nature,* 251, 324 - 327.

Maisterrena, B., Comte, J. & Gautheron, D.C. (1974). *Biochem. biophys. Acta,* 367, 115 - 126.

Makinose, M. & Hasselbach, W. (1971) *FEBS Lett.* 12, 271.

Manson, L.A. & Lampen, J.O. (1951) *J. Biol. Chem.* 191, 95 - 104.

Manteifel, V.M. & Meisel, M.N. (1965) *Fed. Proc.* 196, T 981 - 988.

Martonosi, A. (1968) *J. Biol. Chem.* 243, 71 - 81.

Martonosi, A. (1971) *Biomembranes,* 1, 191.

Martonosi, A., Donley, J. & Halprin, R.A. (1968) *J. Biol. Chem.* 243, 61 - 70.

Martonosi, A. & Halprin, R.A. (1971) *Arch. Biochem. Biophys.* 144, 66 - 77.

Mattoon, J.R. & Balcavage, W.X. (1967) in *"Methods in Enzymol",* 10, 135 - 142.

Maxwell, E. & Ashwell, G. (1953). *Arch. Biochem. Biophys.* 43, 389 - 398.

Mayer, R.J. & Hübscher, G. (1971) *Biochem. J.* 124, 491 - 500.

McGivan, J.D. & Klingenberg, M. (1971) *Eur. J. Biochem.* 20, 392 - 399.

Mela, L. (1968) *Arch. Biochem. Biophys.* 123, 286 - 293.

Meli, J. & Bygrave, F.L. (1972), *Biochem. J.* 128, 415 - 420.

Melnick, R.L., Tinberg, H.M., Maguire, J. & Packer, L. (1973) *Biochim. biophys. Acta,* 311, 230 - 241.

Mendelsohn, J., Skinner, A. & Kornfeld, S. (1971). *J. Clin. Invest.* 50, 818 - 826.

Menzel, H.M. & Hammes, G.G. (1973) *J. Biol. Chem.* 248, 4885 - 4889.

Migala, A., Agostini, B. & Hasselbach, W. (1973). *Z. Naturforsch.* 28c, 178.

Mitchell, P. & Moyle, J. (1967) *Biochem. J.* 105, 1147.

Mitchell, P. (1966), *Biol. Rev.* 41, 445 - 502.

Mitchell, P. (1973), *FEBS Lett.* 33, 267 - 274.

Mitchell, P. (1974), *FEBS Lett.* 43, 189 - 193.

Morrison, J.F. (1968) *Anal. Biochem.* 24, 106 - 111.

Moyle, J. & Mitchell, P. (1975) *FEBS Lett.* 56, 55 - 61.

Mongar, J.L. & Schield, H.O. (1958) *J. Physiol.* 140, 272 - 284.

Morton, R.A.(1961) in *Ciba Symposium, "Quinones in Electron Transport"* p 5. G.E.W. Wolstenhome (ed).

Moore, L., Fitzpatrick, D.F., Chen, T.S. & Landon, E.J. (1974). *Biochim. biophys. Acta.* 345, 405 - 418.

Mukherjee, C. & Jungas, R.L. (1975) *Biochem. J.* 148, 229 - 235.

Mukherjee, S.P. & Lynn, W.S. (1977) *Arch. Biochem. Biophys.* 184, 69 - 76.

Munn, E.A. & Blair, P.V. (1967) *Z. Zellfrsch. Mikroskop. Anat.* 80, 205 - 213.

Munn, E.A. (1976) in *"The Structure of Mitochondria"*, pp 1 - 43., Academic Press INC. (London) Ltd., Oval Road, London.

Naiditch, W.P. & Cunningham, D.D. (1977) *J. Cell. Physiol.* 92, 319 - 332.

Naninga, L.B. (1961) *Biochim. biophys. Acta.* 54, 335.

Newsholme, E.A. & Start, C. (1974) in *"Regulation in Metabolism"*, J. Wiley & Sons, London.

Nordeen, S.K. & Young, D.A. (1977) *J. Biol. Chem.* 252, 5324 - 5331.

Nordlie, R.C. & Lardy, H.A. (1961) *Biochim. biophys. Acta.* 50, 189 - 191.

Novikoff, A.B. (1961) in *"The Cell"* Vol II, p 299, J. Bracher & A.E. Mirsky, (eds), Academic Press, New York.

Ohnishi, T. & Ohnishi, T. (1962) *J. Biochem. (Tokyo)*, 51, 380 - 381.

Ohnishi, T. & Ebashi, E. (1963) *J. Biochem. (Tokyo)*, 54, 506 - 511.

Ohnishi, T. & Asakura, T., Wilson, D.F. & Chance, B. (1972). *FEBS Lett.* 21, 59 - 62.

Ogawa, Y. (1970). *J. Biochem. (Tokyo).* 67, 667 - 683.

Okamoto, H., Sone, N., Hirata, H., Yoshida, M. & Kagawa, Y. (1977) *J. Biol. Chem.* 252, 6125 - 6131.

Orme-Johnson, N.R., Orme-Johnson, W.H., Hansen, R.E., Beinert, H. & Hatefi, Y. (1971) *Biochem. Biophys. Res. Commun.* 44, 446 - 452.

Ostwald & MacLennan, D.H. (1974). *J. Biol. Chem.* 249, 974 - 979.

Ozawa, K., Seta, K., Takeda, H., Ando, K., Handa, H. & Araki, C. (1966) *J. Biochem. (Tokyo)* 59, 501 - 510.

Packer, L. (1957) *Arch. Biochem. Biophys.* 70, 290 - 293.

Packer, L. (1958) *Exp. Cell Res.* 15, 551 - 559.

Parr, D.R. & Harris, E.J. (1976) *Biochem. J.* 158, 289 - 294.

Palade, G.E. (1952) *Anat. Record,* 114, 427 - 451.

Palmieri, F., Prezioso, G., Quagliariello, E. & Klingenberg, M. (1971) *Eur. J. Biochem.* 22, 66 - 74.

Pande, S.V. & Mead, J.F. (1968) *J. Biol. Chem.*, 243, 352 - 361.

Parker, C.W. (1974) *Biochem. Biophys. Res. Commun.* 61, 1180 - 1186.

Parsons, D.F. (1965) *Intern. Rev. Exptl. Pathol.* 4, 1 - 54.

Parsons, D.F., Williams, G.R. & Chance, B. (1966) *Ann. N.Y. Acad. Sci.* 137, 643 - 666.

Parsons, D.F. & Williams, G.L. (1967) *"Methods in Enzymol".* 10, 443 - 448.

Parsons, D.F. & Yano, Y. (1967) *Biochim. biophys. Acta* 135, 362 - 364.

Parsons, D.F., Williams, G.R., Thompson, W., Wilson, D. & Chance, B. (1967). *"Round Table Discussion on Mitochondrial Structure and Compartmentation"* p 20; E. Quagliariello, S. Papa, E.C. Slater & J.M. Tager (eds). Adriatica Editrice, Bari.

Passow, H., Rothstein, A. & Clarkson, T.W. (1961) *Pharmacol. Rev.* 13, 185 - 224.

Passow, H. (1971) *J. Membrane Biol.* 6, 233 - 258.

Peters, J.H. & Hausen, P. (1971) *Eur. J. Biochem.* 19, 502 - 508.

Pedersen, P.L. (1975) *Bioenergetics* 6, 243 - 275.

Pedersen, P.L. (1976) *J. Biol. Chem.* 249, 3579 - 3585.

Peng, C.F., Price, D.W., Bhuvaneswraren, C. & Wadkins, C.L. (1974) *Biochem. Biophys. Res. Commun.* 56, 134 - 141.

Penefsky, H.S., Pullman, M.E., Datta, A. & Racker, E. (1960) *J. Biol. Chem.* 235, 3330 - 3336.

Penefsky, H.S. (1974) *J. Biol. Chem.* 249, 3579 - 3585.

Perrin, D.D. & Sayce, I.G. (1967) *Talanta* 14, 833 - 842.

Pfaff, E. & Klingenberg, M. (1968) *Eur. J. Biochem.* 6, 66 - 79.

Pfeiderer, G. (1965) in *"Methods of Enzymatic Analysis"* pp 381 - 383.

Pickett, W.C., Jesse, R.L. & Cohen, P. (1977) *Biochem. biophys. Acta.* 486, 209 - 213.

Post, R.L., Merritt, C.R. & Kinsolving, C.R. & Albright, C.D. (1960) *J. Biol. Chem.* 235, 1796 - 1802.

Post, R.L., Sen, A.K., Rosenthal, A.S. (1965) *J. Biol. Chem.*, 240, 1437.

Post, R.L. & Sen, A.K. (1967) *Methods Enzymol.* 10, 773 - 776.

Potter, R.L. & Bethel, F.H. (1952) *Fed. Proc.* Vol. 11, p 270.

Pressman, B.C. (1968) *Fred. Proc.* 32, 1698 - 1703.

Puckle, J.L. (1974) *B.Sc. (Hons) Thesis*, Australian National University.

Pullman, M.E., Penefsky, H.S., Datta, A. & Racker, E. (1960) *J. Biol. Chem.* 235, 3322 - 3329.

Pullman, M.E. & Monroy, G.C. (1963) *J. Biol. Chem.*, 238, 3762 - 3769.

Pybus, J. (1969) *Clin. Chim. Acta* 23, 309 - 317.

Quagliariello, E. & Palmieri, F. (1970) *FEBS Lett.* 8, 105 - 108.

Quagliariello, E., Palmieri, F., Prezioso, G. & Klingenberg, M. (1969) *FEBS Lett.* 4, 251 - 254.

Racker, E., Burstein, C., Loyter, A. & Christiansen, R.O. (1970) in *"Electron Transport and Energy Conservation"* Tager, J.M., Papa, S., Quagliariello, E. & Slater, E.C. (eds) pp 235 - 252, Adriatica Editrice, Bari.

Racker, E. (1972). *J. Membr. Biol.* 10, 221 - 235.

Racker, E. & Kandrach, A. (1973) *J. Biol. Chem.* 248, 5841 - 5847.

Racker, E., Chien, T.F. & Kandrach, A. (1975) *FEBS Lett.* 57, 14 - 18.

Raff, M.C., Freedman, M.H. & Gomperts, B. (1975) in *"Membrane Receptors of Lymphocytes"* Seligmann, M., Prund'homme, J.L. & Kourilsky, F.M. (eds) pp 393 - 398 North Holland, Amsterdam.

Ragan, C.I. & Racker, E. (1973) *J. Biol. Chem.* 248, 2563 - 2569.

Rajewsky, B., Gerber, G. & Pauly, H. (1957) in *"Advances in Radiobiology"*. de Hevesy, G.C., Frossberg, A.G. & Abbatt, J.D. (eds) Publ: Oliver & Boyd, Ltd., Edinburgh.

Ramsay, R. & Tubbs, P.K. (1976) *Eur. J. Biochem.* 69, 299 - 303.

Randle, P.J., Denton, R.M., Pask, H.T. & Severson, D.C. (1974). *Biochem. Soc. Symp.* 39, 75 - 88.

Rao, K.N., de Smet, M., Howells, A.J. & Bygrave, F.L. (1974). *FEBS Lett.* 41, 185 - 188.

Racker, E. (1970) in *"Membranes of Mitochondria and Chloroplasts"*, Racker, E. (ed) pp 127 - 212. Van Nostrand Reinhold Company, New York.

Rasmussen, H. (1970) *Science*. 170, 404 - 412.

Rasmussen, H. & Nagata, N. (1970) *Biochim. biophys. Acta*, 215, 17 - 28.

Ray, T.K. (1970) *Biochim. biophys. Acta* 196, 1 - 9.

Reed, K.C. (1972) *Anal. Biochem.* 50, 206 - 212.

Reed, K.C. & Bygrave, F.L. (1974) *Biochem. J.* 140, 143 - 155.

Reed, K.C. & Bygrave, F.L. (1975) *Anal. Biochem.* 67, 44 - 54.

Reed, P.W. & Lardy, H.A. (1972) *J. Biol. Chem.* 247, 6970 - 6977.

Reynafarje, B. & Lehninger, A.L. (1969) *J. Biol. Chem.* 244, 584 - 593.

Rieske, J.S., MacLennan, D.H. & Colemann, R. (1964) *Biochem. Biophys. Res. Commun.* 15, 338 - 344.

Righetti, P. & Cerletti, P. (1971) *FEBS Lett.* 13, 181 - 183.

Ringler, R.L. & Singer, T.P. (1958) *Biochim. biophys. Acta*, 29, 661 - 662.

Ringler, R.L. (1961) *J. Biol. Chem.*, 236, 1192 - 1198.

Roberts, J.B. & Bygrave, F.L. (1973) *Biochem. J.* 136, 467 - 475.

Rossi, C.S. & Lehninger, A.L. (1964) *J. Biol. Chem.* 239, 3971 - 3980.

Rowsell, E.V., Snell, K., Carnie, J.A. & Rowsell, K.V. (1972). *Biochem. J.* 127, 155 - 165.

Roos, D. & Loos, J.A. (1973). *Exp. Cell. Res.* 77, 127 - 135.

Ryrie, I.J. (1975) *Arch. Biochem. Biophys.* 168, 704 - 711.

Sacktor, B. (1977) in *"Current Topics in Bioenergetics"*, Vol. 6, pp 39 - 81, D.R. Sandri (ed); Academic Press, New York.

Sadler, M.H., Hunter, D.R. & Haworth, R.A. (1974). *Biochem. Biophys. Res. Commun.* 59, 804 - 812.

Sandri, G., Panfili, E. & Sottacasa, G.L. (1976) *Biochem. Biophys. Res. Commun.* 68, 1272 - 1279.

Saris, N.E.L. (1963) *Commentat. Biol. Soc. Sci. Fenn.* 28, 1 - 59.

Scaife, J.F. (1966). *Canad. J. Biochem.* 44, 433 - 448.

Scaife, J.F. & Hill, B. (1963) *Canad. J. Biochem.* 41, 1223 - 1233.

Scarpa, A. & Azzone, G.F. (1970) *Eur. J. Biochem.* 12, 328 - 335.

Scarpa, A. & Graziotti, P. (1973) *J. Gen. Physiol.* 62, 756 - 772.

Schick, L. & Butler, L.G. (1969) *J. Cell. Biol.*, 42, 235 - 240.

Schmidt, E. (1974) in *"Methods of Enzymatic Analysis"*, Vol 2, pp 650 - 656.

Schartzmann, H.J. (1967) *Protides Biol. Fluids Proc. Collog. Bruges.* 15, 233.

Schartzmann, H.J. & Rossi, G.L. (1971) *Biochim. biophys. Acta* 241, 379 - 392.

Schnaitman, C.A. (1969) *Proc. Natn. Acad. Sci. U.S.A.* 63, 412 - 419.

Schnaitman, C.A., Erwin, V.G. & Greenawalt, J.W. (1967) *J. Cell. Biol.* 32, 719 - 735.

Schnaitman, C.A. & Greenawalt, J.W. (1968) *J. Cell. Biol.* 38, 158 - 175.

Schnaitman, C.A. & Pedersen, P.L. (1968) *Biochem. Biophys. Res. Commun.* 30, 428 - 432.

Schnebli, H.P. & Abrams, A. (1970) *J. Biol. Chem.* 245, 1115 - 1121.

Scholte, H.R., Weijers, P.J. & Witpeers, E.M. (1973) *Biochim. biophys. Acta*, 76, 764 - 773.

Schuster, S.M. & Olson, M.S. (1974) *J. Biol. Chem.* 249, 7159 - 7165.

Scott, K.M., Hwang, K.M., Jurkowitz, M. & Brierley, G.P. (1971) *Arch. Biochem. Biophys.* 147, 557 - 567.

Sedgwick, B. & Hübscher, G. (1965) *Biochim. biophys. Acta*, 106, 63 - 77.

Senior, A.E. (1973). *Biochim. biophys. Acta.* 301, 249 - 277.

Serrano, R., Kanner, B.I. & Racker, E. (1976) *J. Biol. Chem.* 251, 2453 - 2461.

Seversen, D.L., Denton, R.M., Pask, H.T. & Randle, P.J. (1974). *Biochem. J.* 140, 225 - 237.

Shchipakin, V.N., Chuchlova, E.A. & Evtodienko, Yu.V. (1976) *Biochem. Biophys. Res. Commun.* 69, 123 - 127.

Shamoo, A.E. & Brodsky, W.A. (1972) *Biochim. biophys. Acta.* 255, 220 - 230.

Shamoo, A.E. & Goldstein, E. (1977) *Biochim. biophys. Acta* 472, 13 - 53.

Shamoo, A.E. & Myers, M. (1974) *J. Membr. Biol.* 19, 163 - 178.

Shamoo, A.E., Meyers, M., Blumenthal, R. & Albers, R.W. (1974) *J. Membr. Biol.* 19, 129 - 140.

Shamoo, A.E. & Ryan, T.E. (1975) *Ann. N.Y. Acad. Sci.* 264, 83 - 97.

Shamoo, A.E., Ryan, T.E., Stewart, P.S. & MacLennan, D.H. (1976) *J. Biol. Chem.* 251, 4147 - 4154.

Skulachev, V.P. (1974) *Biochem. Soc. Spec. Publ.* 4, 175 - 184.

Skou, J.C. (1957) *Biochim. biophys. Acta* 23, 394 - 401.

Skou, J.C. (1960) *Biochim. biophys. Acta* 42, 6 - 23.

Skou, J.C. (1965) *Physiol. Rev.* 45, 596 - 617.

Skou, J.C. & Hillberg, C. (1969) *Biochim. biophys. Acta* 185, 198 - 219.

Siliprandi, D., Toninello, A., Zoccarato, F., Rugolo, M. & Siliprandi, N. (1975) *Biochem. Biophys. Res. Commun.* 66, 956 - 961.

Sillen, L.G. & Martell, A.E. (1964) *Chem. Soc. Spec. Publ.* 17, 634 - 641.

Sillen, L.G. & Martell, A.E. (1971) *Chem. Soc. Spec. Publ.* 25, 650 - 654.

Singer, T.P. (1963) in *"The Enzymes"*, Vol. 7, Boyer, P.D., Lardy, H.A. & Myrbäck, K. (eds), pp 345 - 381, Academic Press, New York.

Slater, E.C. (1955) *Biochem. J.* 59, 392 - 405.

Slein, M.W. (1965) in *"Methods of Enzymatic Analysis"* pp 117 - 123.

Smith, L., Davies, H.C., Reichlin, M. & Margoliash, E. (1973) *J. Biol. Chem.* 248, 237 - 243.

Smoly, J.M., Wakabayashi, T., Addink, A.D.E. & Green, D.E. (1971), *Arch. Biochem. Biophys.*, 131, 6 - 21.

Sone, N., Yoshida, M., Hirata, H., & Kagawa, Y. (1975) *J. Biol. Chem.* 250, 7217 - 7923.

Sordahl, L.A. (1974) *Arch. Biochem. Biophys.* 167, 104 - 113.

Sottacasa, G.L., Ernster, L., Kuylenstierna, B. & Bergstrand, A. (1967a) *"Round Table Discussion on Mitochondrial Structure and Compartmentation"* p 20; Quagliarello, E., Papa, S., Slater, E.C. & Tager, J.M. (eds), Adriatica Editrice, Bari.

Sottacasa, G.L., Kuylenstierna, B., Ernster, L. & Bergstrand, A. (1967b) *J. Cell Biol.* 32, 415 - 438.

Sottacasa, G.L., Kuylenstierna, B., Enster, L. & Bergstrand, A. (1967c) *"Methods in Enzymol"*, 10, 448 - 463.

Sottacasa, G.L., Sandri, G., Panfili, E., de Bernard, B., Gazzotti, P., Vasington, F.D. & Carafoli, E. (1972) *Biochem. Biophys. Res. Commun.* 47, 808 - 813.

Spencer, T.L. & Bygrave, F.L. (1973) *Bioenergenetics*, 4, 347 - 362.

Stewart, P.S. & MacLennan, D.H. (1974) *J. Biol. Chem.*, 249, 985 - 993.

Stewart, P.S., MacLennan, D.H. & Shamoo, A.E. (1976). *J. Biol. Chem.* 251, 712 - 719.

Stoffel, W. & Schiefer, H.G. (1968) *Hoppe-Seyler's Z. physiol. Chem.* 349, 167 - 181.

Sugano, T. & Nagai, O. (1971) *J. Biochem. (Tokyo)* 70, 417 - 427.

Sulakhe, P.V. & Dhalla, N.S. (1971) *Life Sciences* 10, 185.

Suter, D. (1973) *Ph.D. Thesis*, Australian National University.

Suter, D. & Weidemann, M.J. (1975) *Biochem. J.* 148, 583 - 594.

Suter, D. & Weidemann, M.J. (1976) *Biochem. J.* 156, 119 - 127.

Swanljung, L., Frigeri, K., Ohlson, & Ernster, L. (1973) *Biochim. biophys. Acta* 305, 519.

Sweat, M.L. (1951) *J. Am. Chem. Soc.* 73, 4056 - 4057.

Szekely, I.E., Fisher, D.R. & Schumacher, H.R. (1976) *Cancer*, 37, 805 - 811.

Tabor, C.W., Tabor, H. & Rosenthal, S.M. (1954) *J. Biol. Chem.* 208, 645 - 661.

Thomson, J.F. (1964) *Radiation Res.* 21, 46.

Thorley-Lawson, D.A. & Green, N.M. (1973) *Eur. J. Biochem.* 40, 403 - 413.

Thorne, R.F.W. & Bygrave, F.L. (1974) *Biochem. J.* 144, 551 - 558.

Tzagoloff, A., Byington, K.H. & MacLennan, D.H. (1968), *J. Biol. Chem.* 243, 2405 - 2412.

Tzagoloff, A., MacLennan, D.H. & Byington, K.M. (1968) *Biochemistry* 7, 1596 - 1610.

Tzagoloff, A. & Meager, J. (1971) *J. Biol. Chem.* 246, 7328.

Vainio, H., Mela, L. & Chance, B. (1970) *Eur. J. Biochem.* 12, 387 - 391.

Vallieres, J., Scarpa, A. & Sombyo, A.P. (1975) *Arch. Biochem. Biophys.* 170, 659 - 669.

Vambutas, V. & Racker, E. (1965) *J. Biol. Chem.* 240, 2660 - 2667.

Van Bekkum, D.W. (1955) *Biochim. biophys. Acta,* 16, 437 - 438.

Van Bekkum, D.W. (1956) in *Ciba Found. Symp.: "Ionising Radiations and Cell Metabolism"* Wolstenholme, G.E.W. & O'Connor, C.M., (eds.), J. & A. Churchill, London.

Van den Berg, S.G. (1967) in *"Methods in Enzymol"*, 10, 105 - 110.

Van de Stadt, R.J., de Boer, B.L. & Van Dam, K. (1973). *Biochim. biophys. Acta.* 292, 338 - 349.

Vasington, F.D. & Murphy, J.W. (1962) *J. Biol. Chem.* 237, 2670 - 2677.

Verity, M.A. & Gambell, J.K. (1968) *Biochem. J.* 108, 289 - 295.

Vignais, P.V., Dull, E.D., Vignais, P.M. & Huet, J. (1966) *Biochim. biophys. Acta.* 118, 465 - 483.

Vinogradov, A., Scarpa, A. & Chance, B. (1972) *Arch. Biochem. Biophys.* 152, 646 - 654.

Vijayakumar, E.K. & Weidemann, M.J. (1976) *Biochem. J.* 160, 383 - 393.

Vijayakumar, E.K. & Weidemann, M.J. (1977) *Biochem. J.* 165, 355 - 365.

Wainio, W.W., Laskowska-Kuta, T., Roseman, J. & Greber, D. (1973) *Bioenergetics,* 4, 453.

Walter, H. & Hasselbach, W. (1973) *Eur. J. Biochem.* 36, 110 - 119.

Wang, T., Marquadt, C. & Foker, J. (1976) *Nature.* 261, 702 - 705.

Weber, A. (1971) *J. Gen. Physiol.* 57, 50 - 63.

Weber, A., Herz, R. & Reiss, I. (1966) *Biochem. Z.* 345, 329 - 369.

Weber, A. (1966) *Curr. Top. Bioenerg.*, 1, 203 - 254.

Weidemann, M.J. & Krebs, H.A. (1969) *Biochem. J.* 112, 149 - 165.

Weidemann, M.J., Erdelt, H. & Klingenberg, M. (1969) in *"Inhibitors - Tools in Cell Research"*, pp 324 - 332, Springer-Verlag, Berlin, Heidelberg and New York.

Weidemann, M.J., Heldt, H. & Klingenberg, M. (1970), *Eur. J. Biochem.* 16, 313 - 335.

Weidemann, M.J., Peskar, B.A., Wrogemann, K., Rietschel, E.Th., Staudinger, H. & Fischer, H. (1978). *FEBS Lett.* (in press).

Whitesell, R.R., Johnson, R.A., Tarpley, H.L. & Regen, D.M. (1977) *J. Cell Biol.* 72, 456 - 470.

Whitney, R.B. & Sutherland, R.M. (1972) *Cell Immunol.* 5, 137 - 147.

Whitney, R.B. & Sutherland, R.M. (1973) *J. Cell. Physiol.*, 82, 9 - 20.

Wieland, O.H., Patzelt, C. & Löffler, G. (1972) *Eur. J. Biochem.* 26, 426 - 433.

Williamson, D.H., Mellanby, J. & Krebs, H.A. (1962) *Biochem. J.* 103, 514 - 527.

Wlodawer, P., Parson, D.F., Williams, G.R. & Wojtozak, L. (1966) *Biochim. biophys. Acta.* 128, 34 - 47.

Wohlrab, H. (1975) *Fed. Eur. Biochem. Soc. Meet.* 10th, Abstract 1114.

Woodin, A.M. & Wienke, A.A. (1963). *Biochem. J.* 87, 487 - 495.

Wray, H.L., Gray, R.R. & Olsson, R.A. (1973) *J. Biol. Chem.* 248, 1496 - 1498.

Wray, H.L. & Gray, R.R. (1977) *Biochim. biophys. Acta*, 461, 441 - 459.

Wu, R. & Sauer, L.A. (1967) in *"Methods in Enzymol."* 10, 117 - 118.

Yago, N. & Ichii, S. (1969) *J. Biochem. (Tokyo)* 65, 215 - 224.

Yashida, M., Sone, N., Hirata, H. & Kagawa, Y. (1975). *J. Biol. Chem.* 250, 7910 - 7916.

Yasmeen, D.E., Laird, A.J., Hume, D.A. & Weidemann, M.J. (1977) *Biochim. biophys. Acta.* 500, 89 - 102.

Yates, D.W. & Garland, P.B. (1966) *Biochem. Biophys. Res. Commun.* 23, 460 - 465.

Yu, C.A., Yu, L & King, T.E. (1972) *J. Biol. Chem.* 247, 1012 - 1019.

Zweig, G. & Sherma, J. (1972) in *"Hand-Book of Chromatography"*, Vol. 1, pp. 411 - 412, Chemical Rubber Company, Ohio.